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STUDIES ON THE GROWTH AND EXTRACELLULAR PRODUCTS OF AN ENTEROTOXIGENIC STAPHYLOCOCCUS

by

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ABSTRACT

The effects of sodium chloride concentration, time and temperature of incubation on the growth and toxin production of an enterotoxin producing staphylococcus were investigated, using serological and electrophoretic methods.

Cultures were grown in broth culture and sac culture. It was found, using a serological gel diffusion test, that with increasing sodium chloride concentrations from 0-12%, that growth and formation of enterotoxin in sac culture is progressively inhibited. This effect was more inhibitory to toxin production than growth. Confirmation of this was obtained by acrylamide gel, disc electrophoresis of the samples. These results are discussed.

The use of disc-gel electrophoresis as a method for the detection of enterotoxin in broth cultures is reported.

Studies on the effect of sodium chloride and temperature on the production of extracellular products other than enterotoxin are described.

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STUDIES ON THE GROWTH AND EXTRACELLULAR PRODUCTS OF AN ENTEROTOXIGENIC STAPHYLOCOCCUS

GENERAL INTRODUCTION

Methods by which microorganisms induce food poisoning may be divided into two categories.

1. The infectious type, that follows the multiplication within the body of pathogenic organisms conveyed by food.
2. The toxic type that follows the ingestion of the food in which poisonous substances have been formed as a result of bacterial proliferation.

Examples of pathogenic organisms capable of producing the first category, the infectious type, are certain strains of the genera Salmonella and Shigella. The toxic form of food poisoning, may be caused by certain strains of Staphylococcus and Clostridium.

Many food poisoning outbreaks appear to be the result of exposure of contaminated food for a few hours at temperatures favourable to the multiplication of bacteria. The control of temperature during processing, storage, and preparation of perishable foods is therefore recognised to be the most dependent and practicable measure of preventing food poisoning outbreaks

STAPHYLOCOCCAL FOOD POISONING

The ability of some strains of staphylococci to produce a toxic substance capable of causing irritation to the gastro-intestinal tract, was not fully recognised at the beginning of the century, due to the belief held that bacterial food poisoning was due entirely to Salmonella

infections. Since then staphylococci have been shown to be probably the most common food poisoning organisms, often drawn to public attention by the large numbers of people involved in a single outbreak. Whether all staphylococci are capable of elaborating the substance causing symptoms of food poisoning is a matter of importance in work associated with the isolation and detection of the causative organisms in cases of food poisoning. Studies of the biochemical characteristics of pathogenic and saprophytic strains, reveal a correlation between coagulase and toxin production (1). There is evidence that a few exceptions to this rule exist (2, 3). The following criteria are considered to be indicative of a potentially pathogenic strain of *Staphylococcus aureus* (4):

Respiration	Fermentation	Coagulase
Facultatively anaerobic	Mannitol +	Coagulase +

Recent findings show no phage pattern associated with the ability of strains to cause food poisoning, except possibly in group IV.

Staphylococcal food poisoning has followed the consumption of a wide range of foods, the only common character of which appears to be the ability to support vigorous growth of the organisms. Little is known of the optimum conditions for enterotoxin production. Contamination by toxin-producing staphylococci is common, due to the widespread occurrence of these organisms in the nasopharyngeal region and on the hands of healthy persons.

Over two-thirds of the reported outbreaks in England and Wales appear to be associated with meat. Invariably this is processed meat.

In the United States, meat and confectionary cause one-third of the outbreaks, and in both countries, dairy-products about 10% of the total cases (5).

The processed meats that give rise to most of the British outbreaks have been prepared by methods in which handling after cooking is inevitable. The most important group includes various forms of pressed, jellied or sliced meats. In the United States ham accounts for nearly a half of the reported outbreaks due to meat.

Cases associated with canned foods are common. Most are due to contamination of the contents after opening, but in some, obvious leakage of the cans during the cooling process through minute defects in the seam has caused spoilage. Most outbreaks are concerned with canned meat, but sardines in oil (6), and shell-fish (7) have been implicated. The normal flora of salt water fish does not include staphylococci, but contamination may occur after hand-filleting of the fish.

Confectionary, in the form of cake fillings such as custard, are a common source of staphylococci. Synthetic creams are not involved in many outbreaks. Dairy products, such as raw milk from cow, goat or human, may under certain conditions support the growth of enterotoxin-producing organisms. Cheese and spray-dried milk powder manufactured from contaminated milk, have been responsible for a number of outbreaks. In these products, the numbers of organisms present in a sample are often scanty or absent and have presumably died out after toxin formation. One of the problems associated with food containing toxin, is that it may appear organoleptically normal.

DIFFUSABLE PRODUCTS OF STAPHYLOCOCCI

The exotoxin includes the following extracellular proteins and enzymes:- α, β , and δ hemolysins, coagulase, enterotoxins, hyaluronidase, protease and lipase.

I. Hemolysins.

Three different hemolysins are recognised by their comparative ability to hemolyze erythrocytes from different animal species and by their serological specificity.

The alpha hemolytic reaction is characterised by rapid lysis of sheep or rabbit erythrocytes when incubated at 37°C, but with little extension of lysis following further incubation at 4°C. Beta-hemolysin is unable to lyse either rabbit or sheep erythrocytes at 37°C, but will cause lysis of sheep cells upon further incubation at 4°C. This feature, specific to beta-hemolysin, is known as the 'hot-cold' reaction.

A hemolysin not neutralised by either α or β antitoxin, and acting on human, monkey, horse, rat, mouse, sheep and rabbit erythrocytes, is named delta-hemolysin.

Only three antigenically distinct hemolysins seem to be produced by coagulase-positive strains.

Alpha-hemolysin has a molecular weight of 44,000, an isoelectric point of 6.4 (8) and has been designated a protein, a proteolytic enzyme or an enzyme (9, 10). Its classical biological effects are lethality, dermonecrosis and *in vitro* hemolysis of sheep erythrocytes.

The toxin is produced with a high hemolytic activity when grown in shake cultures of brain heart infusion broth with an atmosphere of 10% CO_2. However, hemolytic activity can also be detected without CO_2 (11). Toxin is produced during periods of multiplication. During the first four generations the toxin/coccus ratio increases, thereafter it remains constant (12). It is known that appreciable autolysis of the organisms occurs during the growth period (13), but whether lysis is needed for toxin release has not been established. According to some workers, the capacity to produce toxin may depend upon the presence of a specific prophage (14).

During starch electrophoresis the α toxin migrates toward the cathode (15). In column chromatographic separations of crude extracellular products of staphylococci, quite often, all of the major peaks appear to be α toxins as they all exhibit the classical biological effects of α toxin. The literature is replete with whether the diverse biological effects of α toxin, (hemolysis, lethality and dermonecrosis) are due to a single substance. The 'unitarian' theory that α toxin is a single toxin, is at present mainly agreed upon but the electrophoretic heterohomogeneity of the α toxin indicates that sub-species may exist.

The chemical composition of beta hemolysin has not been studied. Similarities in its mode of action to that of α hemolysin suggest it to be enzymatic in nature. It is characterised on sheep's blood at 37°C by a zone of discolouration which clears when kept at 4°C.

Production of the toxin in a medium of brain heart infusion and yeast extract in shake cultures at 37°C produces maximum activity

(16). Rapid inactivation of β toxin will occur in broth cultures if held for too long at room temperature (17). For purification purposes, a strain of organism producing β hemolysin only is recommended, as alpha and beta hemolysins appear to exhibit a mutual antagonism to each other. A synergism exists between β and δ hemolysins (18).

Delta-hemolysin is the only hemolysin able to attack human erythrocytes. It is found that the sera of most normal people contain large amounts of neutralising antibody.

Delta-hemolysin exhibits considerable heat stability, little change in titre occurring when heated at 65°C for two hours (19).

II. Coagulase.

The literature concerning coagulase is voluminous, and many problems still exist. A brief review of some of the latest findings indicates that coagulase has a molecular weight of 44,000 (20) and is possibly protein in nature. There may be differences structurally and antigenically in the coagulase molecule between species of staphylococci (21).

The ability to clot plasma as an expression of its activity, leads to the assumption that coagulase may be an enzyme. However, enzymatic actions such as lipase, deoxyribonuclease and phosphatase have been eliminated (22) and there seems no convincing evidence that enzymatic activities of purified coagulase can be equated with clotting ability.

III. Hyaluronidase

Of the coagulase positive staphylococci 93.6% produce hyaluronidase (23). Hyaluronidase is an enzyme, and is formed in increasing amounts for the first six days of incubation. Compared to coccal multiplication there is a lag period, during which no hyaluronidase is formed. The actual mode of production of the enzyme by staphylococci is not known.

The action of hyaluronidase is primarily as a spreading factor involved in the invasiveness of the cocci, hydrolysing the mucoid ground substance of the connective tissue and thus removing the physical obstacle leading to spread of the invading organism.

IV. Minor extracellular products.

The function of extracellular enzymes is to break down available nutrients into simpler units readily utilisable by the organism. Hence according to the substrate, an organism will release into the medium various enzymes such as proteases, lipases and hydrases. In the case of pathogenic micro-organisms the extracellular enzymes are the first interaction between host and parasite.

V. Enterotoxin.

A major area of conflicting evidence was previously centered upon whether food poisoning symptoms in animals and man were caused by a specific enterotoxin or by either α or β hemolysins. As recently as 1959, it was noted that a culture filtrate which contained hemolysin

could induce *in vitro* gut stimulation (24). However, a non-hemolytic, non-dermonecrotic, non-lethal toxin has been isolated from the filtrates of food-poisoning strains, distinguishing it from the lethal action of the lysins (25). This supports evidence that antisera neutralising the classical toxins α or β fails to affect enterotoxin (26, 27). The enterotoxin therefore, appears to be the emetic substance associated with staphylococcal food poisoning.

It has been reported that at least four serologically distinct types of enterotoxin occur (28, 29, 30, 31). They are closely related and have been designated enterotoxin A, B, C and D respectively (32).

Investigation, in terms of production of emesis on oral administration of toxin, reveals that all enterotoxins give rise to identical symptoms. Epidemiologically however, staphylococci producing type A enterotoxin are most frequently isolated from foods (33). This is probably associated with the relative incidence of staphylococci producing A type toxin isolated from human sources. Recently cases implicating enterotoxin B in human food poisoning have been reported from Japan (34). It is also known that the majority of strains producing B also produce a small amount of A. Quite often this combination can produce symptoms other than common food poisoning, for example, enterocolitis (35).

(i) Site of enterotoxin production.

Techniques, such as the immunofluorescent identification of antigens, have been used to demonstrate the cellular location of the enterotoxins in a culture of toxin-producing staphylococci (36). The

failure to demonstrate an accumulation of enterotoxins in cells of these organisms on examination of lysates led to morphological investigations of the cells. Observations indicate that enterotoxins are present as a loose binding on the surface of the bacterial cell, probably being formed as a metabolic product and passed out of the cell into the growth medium (37).

The nature of the enterotoxins has until recently been unknown due to inadequate purification procedures. At present enterotoxin A has been purified to 70% (38) and enterotoxin B to 99% (39). Most current literature is concerned with the chemistry, physical, and pharmacological properties of enterotoxin B.

(ii) Chemical composition of Enterotoxin B.

Pure, freeze dried toxin has the appearance of a fluffy, white powder, extremely soluble in water and having a toxicity ED_{50} of 0.1 mg/kg in rhesus monkeys.

Recent studies of the amino acid composition of enterotoxin B have shown that it is a simple protein containing approximately nineteen amino acids (40). The toxin is devoid of lipids, carbohydrates and nucleic acids, and the active principle of crude toxin has been shown to be undialysable (41). The molecular weight of the protein is estimated at 24,000 (42), 35,000 (43) with an isoelectric point of 8.7 (40). It is particularly rich in aspartic acid and lysine, the two comprising one third of the total number of residues and weight of the protein. Glutamic acid and tyrosine are also present in abundance (44).

There are no free sulphydryl groups and only one disulphide bridge, accounting for ten methionine residues and two and half cystine residues. A single amino-terminal residue and a carboxy-terminal residue have also been shown to be present. In attempts to isolate the biologically active portion of the protein, several workers have tried to eliminate possible constituents (45). The single disulphide bridge, when reduced, results in the freeing of sulphydryl groups, which, on alkylation with iodacetamide, produce derivatives with the same emetic activity and immunological properties as control toxin. Only slight alteration of the physical properties occur. Thus it seems that information regarding the formation of biologically active enterotoxin resides in the amino-acid sequence.

The simplest and most probable structure satisfying all these conditions, is a single polypeptide chain, although a two-chained molecule has been considered. The latter structure may dissociate in the intestinal tract and free small toxic units that could pass through the gut-wall intact. As data indicate that the toxin is resistant to the action of pancreatic endopeptidases, this dissociation would circumvent the problem of adsorption of a large protein molecule. However it seems unlikely that such a double chain could be bound together by a disulphide bridge, or by non-covalent bonds.

Tending to complicate the situation, is the fact that the protein is resolved into two main protein components by electrophoresis (46).

The picture of a water-soluble protein, rich in lysine, resistant to trypsin, and possessing antigenic properties, tends to support

the view that enterotoxins have the characteristics of a cell wall surface constituent being distinct, chemically, physically and biologically from the endotoxins.

(iii) Stability of enterotoxin B.

In outbreaks of food-poisoning, especially those caused by dried products such as skim-milk powder, few implicating organisms are detected. This shows that toxin formed prior to heat treatment must be heat-stable. Data indicates that the pure toxin is heat-resistant, and able to withstand boiling for thirty minutes (47). This treatment reduces, but does not destroy its activity. Evidence indicates that crude filtrates are more heat-stable than purified toxins (48) and that the other staphylococcal toxins are less heat-resistant than the enterotoxins. Recent experiments studying the thermal inactivation of enterotoxin B in milk, indicate that the time/temperature combinations used in normal pasteurisation, sterilisation and spray drying of raw milk, are insufficient to reduce the level of toxin below that causing emesis in humans (49).

Partially purified enterotoxin is known to be able to withstand storage at 37°C for at least eight months and crude filtrates for three months at 4°C, without apparent loss in activity.

Isoelectric studies with the toxin reveal a tolerance to a fairly wide pH range. It will withstand pH 3-12 for four hours at room temperature (50). A combination of pH and temperature which has been found to give 95% inactivation, is pH 6 at 100°C for at least 30 minutes.

Summarizing, it can be seen that staphylococcal enterotoxin B is an extremely stable substance.

(iv) Mode of action of enterotoxin B.

Mechanism of action of staphylococcal toxin resulting in the food poisoning syndrome, is not well understood. Symptoms of nausea, retching, vomiting, abdominal cramping and diarrhoea appear one to four hours after ingestion of the toxin. The incubation period depends not only on the amount of toxin ingested but on the individual affected. Some individuals have been reported to be completely unaffected by enterotoxin ingestion. It is known that in animals the degree of susceptibility decreases after two or three inoculations. Some form of immunity must be developed. Results suggest that resistance to enterotoxin must be due to the presence of antibodies in the serum of resistant animals. An interesting study showed that over 20% of food sold in open markets in Thailand was contaminated by enterotoxic strains of staphylococci. Examining serum for antitoxin levels, it was found that values for permanent residents were higher than visitors to Thailand (51). Immunization of monkeys with formaldehyde-treated toxins has been attempted, but the results were not specific enough for tests on human volunteers to be made (52).

Vomiting produced by enterotoxin is a reflex action requiring a high degree of coordination involving many factors. Two channels of thought are apparent in connection with the site and mode of emetic action.

1. Toxin acts on the peripheral sensory structures of the gut, and is therefore a form of neurotoxin (53).

2. Direct action of toxin upon the smooth muscle of the gut (54).

Recent work, using test-animals, has indicated that the enterotoxin is not a neurotoxin, but that the site of its emetic action is on the abdominal viscera. The sensory stimulus for vomiting reaches the vomiting centre by way of the vagus and sympathetic nerves (55).

Studies of the direct action of the toxin upon the human and monkey gastric mucosa, suggest that enterotoxin acts on the jejunal mucosa causing a change in the cytology of the cells. Inflammation and epithelial damage occur two to four hours after toxin injection. Lipid droplets are seen to disappear from the cells, indicating an alteration in the pattern of fat absorption (56, 57). These symptoms rapidly regress and the mucosa becomes nearly normal after 72 hours. This time interval, corresponds approximately with the usual period of distress associated with food poisoning.

Similarities between the host responses caused by endotoxins of staphylococci and enterotoxins have been examined (58). Whereas the enterotoxin is protein in nature, endotoxin is usually a lipopolysaccharide, and forms part of the complex associated with the fine structure of the cell wall. Symptoms caused by endotoxins when injected into animals are characterised by biphasic changes in the phagocytic functioning of the reticulo-endothelial system. Similarly, a blockade of this system has been observed by injection of enterotoxin associated with an apparent increase in the

susceptibility towards emesis. This could be a direct endotoxin-like property, or, could result from an indirect mechanism. Enterotoxin could alter the gut so that the endotoxins of the indigenous microflora of the gastro-intestinal tract, would be able to exert their toxic actions. Another explanation is a tissue-enterotoxin interaction, producing toxic products with potentialities for evoking the endotoxin-like responses, for example proteolytic enzymes. The protective action of a fraction of Australian tiger-snake venom, against the effects of staphylococcal toxin, suggest protection due to prior attachment to sites of action of the toxin (59). This would seem to indicate some form of enzyme-like involvement.

CONDITIONS FOR GROWTH OF STAPHYLOCOCCI IN FOOD

A number of conditions must be fulfilled if staphylococcal food poisoning is to occur. First, an article of food has to be sufficiently contaminated with an enterotoxin-producing strain of the organism. Second, the food must be stored for a sufficient length of time at temperatures which encourage multiplication. Third, the food must be a good medium for the growth of organisms and subsequent toxin production. On the basis of these considerations, preventative measures may be devised.

I. Sources of contamination

A large percentage of the population are known to be nasal carriers of toxin-producing staphylococci. Cuts on hands can lead to

contamination of foods requiring handling, especially meats, confectionary and dairy products.

Milk may become contaminated from either bovine or human sources. Cows showing clinical or subclinical forms of mastitis, due to infections of S. aureus, are found to excrete these organisms into the milk (60).

II. Conditions of treatment and storage

(i) Effects of temperature.

Evidence is available that temperatures of less than 10°C are necessary to inhibit the development of staphylococci in foods. Slow growth at 6°C has been reported and data indicate that growth is prevented when the internal temperature of the foodstuff is at, or below, 4°C (61).

Optimum temperatures at which staphylococci are capable of producing enterotoxin appear to be between 25°C and 35°C (62).

Staphylococci are capable of growing in certain foods up to a temperature of 45°C. Death occurs after 24 hours storage at 47°C (63). Recommended heat treatment for foods based on thermal death studies, would indicate 45 minutes at 60°C, or 12 minutes at 77°C as adequate for reducing the total microbial count by 10 million staphylococci/gram (64). In practice, exposure times of longer duration at 77°C would provide a safety factor. Certain articles, such as potato salad, after heat treatment cool very slowly when placed in a refrigerator and allow enormous growth of bacteria (65).

Food poisoning outbreaks involving coagulase - positive staphylococci have frequently incriminated cheese made from raw milk, milk subjected to subpasteurisation temperatures, and milk exposed to postpasteurisation contamination. Comparing the thermal resistance of S. aureus in milk, whey, skim and buffer, differences in the protection from heat afforded to staphylococci by the supporting media have been found (66). A culture in the stationary phase of growth in milk required 4 minutes at 60°C for 99.999% inactivation, whereas in skim milk it took 7 minutes and in whey 6.5 minutes to be inactivated. Actively growing cultures, however, require 60° for 12 minutes in whole milk and 60° for 12 minutes for skim milk. Pasteurisation of whole milk for liquid consumption would seem to adequately destroy any staphylococci present, but at 55°C for 20 - 30 seconds, a method of 'flash-treating' milk for raw milk cheese, pathogenic bacteria may survive.

As previously noted, enterotoxin is fairly heat-resistant. It has been shown that although pasteurisation is sufficient to kill the organisms themselves, it is not sufficient to destroy the preformed toxin. Being facultative anaerobes, staphylococci are also capable of growth and enterotoxin production to a certain extent under anaerobic conditions.

(ii) Competitive microorganisms

It has long been observed that cooked meat is implicated in food-poisoning outbreaks more often than raw meat. As a result of the cooking process the natural flora of the meat, being less heat-resistant

than the staphylococci, is killed, giving the latter opportunity to grow more rapidly.

Similarly in milk of poor bacteriological quality competition from saprophytic organisms, especially faecal and lactic streptococci, appears to restrain the growth sufficiently to prevent formation of toxin (67).

In the case of frozen cooked vegetables, as well as the saprophytic population providing protection, the food product usually becomes organoleptically unsuitable before appreciable staphylococci can arise.

It seems likely therefore, that the majority of cases of food poisoning occur in food which has been treated to reduce bacterial population by heat treatment. Certain foods are also known to have a protective action toward staphylococci, such as eggs, starches and lipids (68). Thus the role of saprophytic organisms in preventing toxin formation in foods is apparent. Maximum inhibition occurs between 20°C and 25°C (69, 70). The mode of action of saprophytic organisms in the inhibition process may take two forms at these temperatures:- (a) a marked effect on staphylococcal growth itself, and hence no enterotoxin, (b) inhibition of enterotoxin production with no apparent affect on growth. The first form of inhibition may be due to the formation of an antibiotic-like substance by competing organisms. The second type could be due to competition for an essential nutrient required for enterotoxin synthesis, or, a breakdown of enterotoxin by microorganisms. It may be, that the antibiotic substance could inhibit

enterotoxin formation but not growth. Antibiotic substances inhibitory to S. aureus have been isolated from Escherichia coli and Bacillus cereus (71).

It can now be said that the presence in food of large numbers of potentially pathogenic staphylococci is not sufficient proof of the presence of enterotoxins. An earlier hypothesis to account for this apparent blocking of enterotoxin formation, is that the toxin may be present in a form which requires further activation, perhaps by specific enzymes or prosthetic groups before being functional (72). This would be similar to the proteolytic activation of botulinum toxin, type E.

III. Food as a medium for staphylococcal growth

Factors associated with the internal environment of food which affect staphylococcal growth are, availability of nutrients, moisture content, and salt concentration. Any food containing optimum concentration of these factors is theoretically capable of supporting growth.

(i) pH

The pH of the food has a direct influence on bacterial growth. Staphylococci grow well at pH 6 - 8 but fail to grow outside this range. Fruit has rarely been implicated in food poisoning, possibly due to its acid reaction.

(ii) Moisture content.

Three traditional methods of food preservation, salting, syruping and dehydration are based on the lowering of moisture content in the foodstuff. Bacteria are unlikely to grow in dehydrated food if the moisture content is below 15%. The actual water requirements of bacteria are least at temperatures and pH values close to the optimum for growth. Some articles such as canned salmon do not become poisonous even after experimental infection with staphylococci (73). Growth and toxin production will occur in pork at 30%, and in beef at 40% and 60% moisture content (74).

(iii) Salt concentration.

Staphylococci are known to be particularly resistant to high salt concentrations, being able to multiply in up to 12% salt, and to survive in up to 25% salt (75).

High salt tolerance, has been associated with the manifestation of a low water requirement by the staphylococcal bacterium (76). Hence their preferential ability to survive, over saprophytic organisms, in environments of low available water such as would exist in a high salt-containing foodstuff. Physiologically the effect of a concentrated salt solution is to reduce the water activity within the cells and hence increases the concentration of intracellular solutes (77). Enzymes of true halophiles are themselves halophilic, but little is known of the activities of enzymes in staphylococci. Reduction of water activity

within the cell leads to a decrease in protein and inorganic phosphate, but the steps in metabolism primarily affected are unknown.

Contamination of foods could occur from dry salt and curing brines, in the case of meat. The preserving action of salt also tends to remove oxygen from solution by reducing the solubility of the gas, thus creating partially anaerobic conditions, favouring the growth of staphylococci. The bacteriostatic action of salt will actually increase with a decrease in temperatures from 21°C to 10°C, due to the accompanying increase required in available water for the growth of organisms at these lower temperatures.

Certain foodstuffs, especially salted meats cured in brines, are more susceptible to staphylococcal contamination and growth than unsalted food. The interface between meat and brine will be favourable to staphylococcal survival as inhibition of saprophytic organisms will occur at high salt concentration (78).

Vacuum-packed bacon slices have been investigated as possible sources of food poisoning. S. aureus is inhibited by this form of packaging but not completely prevented from growing (79), mainly due to its ability to grow at the lowered water activity levels present in the bacon after brine curing. The organism's ability to grow anaerobically is also an important factor.

Studying the possibilities of enterotoxin formation in sliced bacon, it was found that under certain conditions, enterotoxin may be formed (80). Thus this form of product may be regarded as a potential hazard. No precise information on enterotoxin production at various salt concentrations is available.

DETECTION OF STAPHYLOCOCCAL ENTEROTOXINS

The detection of enterotoxins under research conditions, and under routine examination, are two different problems. Time and cost are of primary importance in the latter case, as it may be essential to condemn or release large quantities of food. A simple and quick test has not been fully developed for routine purposes, and at present the number of coagulase-positive staphylococci found in a "suspect" sample is taken as an indication of contamination. Many types of differential media selective for such staphylococci have been developed (81). Difficulties arise with such foods as cheese and milk powder, where, as stated before, the number of organisms may be very much reduced and the toxin still remain. In a previous discussion it was also noted that the number of staphylococci present in any foodstuff is no indication of the presence of enterotoxin.

I. Animal tests.

The first techniques based on this idea were concerned with the feeding of whole cultures or filtrates from the incriminated food sample into the abdominal cavity or blood stream of animals (82). It was known that provided the α and β hemolysins were destroyed by heat, trypsin-action (83) or by antiserum, before injection into the test animal, the enterotoxin could be demonstrated by its ability to give rise to vomiting and diarrhoea in 6 - 8 week old kittens. Some doubt as to how far the "kitten test" may be regarded as a specific reaction to enterotoxin has since been raised. Several workers report

non-specific reactions after the intraperitoneal injection of control materials, strong brines, and mustard oils (84, 85). Others point out that unless the sample is boiled for over 15 minutes, some residues of a large initial content of β hemolysin may remain and cause a positive reaction.

It seems to be a matter of conflict as to whether the rhesus monkey (86), kitten (87) or man, are the most reliable. Unfortunately all have disadvantages. Monkeys, for example, are expensive to keep, develop immunity quickly, and are highly variable in their response to the emetic principle. In general the biological tests available are of variable reliability.

II. Serological tests.

Crude extracts of staphylococcal broth filtrates are known to contain numerous antigenic materials, each one producing a corresponding antibody. Purification of enterotoxins A and B, and the determination that they are antigenically active proteins (88) and (89) has given rise to the preparation of specific monovalent sera. This has proved of use in assaying the toxins *in vitro* by means of such immunological techniques as the Oudin gel diffusion test (90), Ouchterlony test (91), Oakley test (92) or Crowle's modified microslide test (93). Each of these is based on the principle that; "diffusion of mixtures of antigens into a gel containing mixtures of their antibodies will produce numerous zones of precipitation". Each antigen having a specific antibody and therefore a specific zone.

The consideration of a practical serological test for staphylococcal enterotoxin, and methods developed of extracting the enterotoxin from the implicated foodstuff, has led to the development of serological tests for the detection of toxin in a contaminated sample. Indications that the problem would not be complicated by an excessive number of types of enterotoxin has been shown (94) and tests for both enterotoxins A and B in foods have been made possible (95).

Extracts of enterotoxin are achieved by homogenisation of food with phosphate buffered saline. On centrifugation, the supernatant is used as the crude antigen preparation (96). As little as 1 µg of antigen can be detected from such preparations by the Oudin and Crowle tests. This sensitivity is critical enough to detect an emetic dose of toxin. Serological methods for detecting enterotoxins in milk and cheese are also reported (97, 98).

Another serological method is a flotation antigen-antibody system (99).

Recently, detection of the toxin precipitated around the outside of living staphylococcal cells has been achieved using immunofluorescent antibodies (100). Concentrations of 1 µg/ml in food smears have been detected by introducing specific antiserum conjugated with isothiocyanate. No information is given on whether toxin, remaining in food extracts after the death of the bacterial cells, can be detected.

The reliability and sensitivity of the serological tests are far superior to those of the animal tests. However, one disadvantage

is that pure toxin is required for the production of specific antiserum. Preparation of reasonably pure toxin is a complex procedure (101), too elaborate for the normal public health laboratory. The production of antiserum therefore becomes a problem, unless it becomes available commercially.

III. Other tests.

Suggestions for detection tests requiring neither antiserum nor animals, have made use of infra-red spectroscopy (102), and indirect hemagglutination inhibition (103).

Examining the boiled filtrates from a nontoxigenic and an enterotoxigenic strain of staphylococci by infra-red spectrophotometry, it is found that at one point the absorption band is always higher in enterotoxin-containing filtrates. Possible use of this technique as a means of detecting enterotoxin has been considered.

From studying the literature, it appeared that the conditions of growth for pathogenic staphylococci in broth cultures and food had been thoroughly investigated. However, the production of enterotoxin had merely been presumed to run parallel with growth of the organisms.

It was decided to investigate some of the variables which may influence enterotoxin formation in broth cultures, with the idea that information obtained could be of use in future investigations concerning toxin production in foods.

The variables time, temperature, and salt concentration were studied in pure cultures of staphylococci growing in broth.

Salt concentration was chosen, as it seemed that at the higher concentrations of salt, staphylococci were able to compete and overgrow saprophytic organisms, especially it seems, in such commodities as ham and bacon. Thus, they are able to achieve a high concentration of organisms, with the possibility of toxin being produced.

The influence of salt on the production of enterotoxin was investigated primarily. However, data on the production of other extracellular products of staphylococci under these conditions has been included.

MATERIALS AND METHODS

MATERIALS AND METHODS

TEST ORGANISM

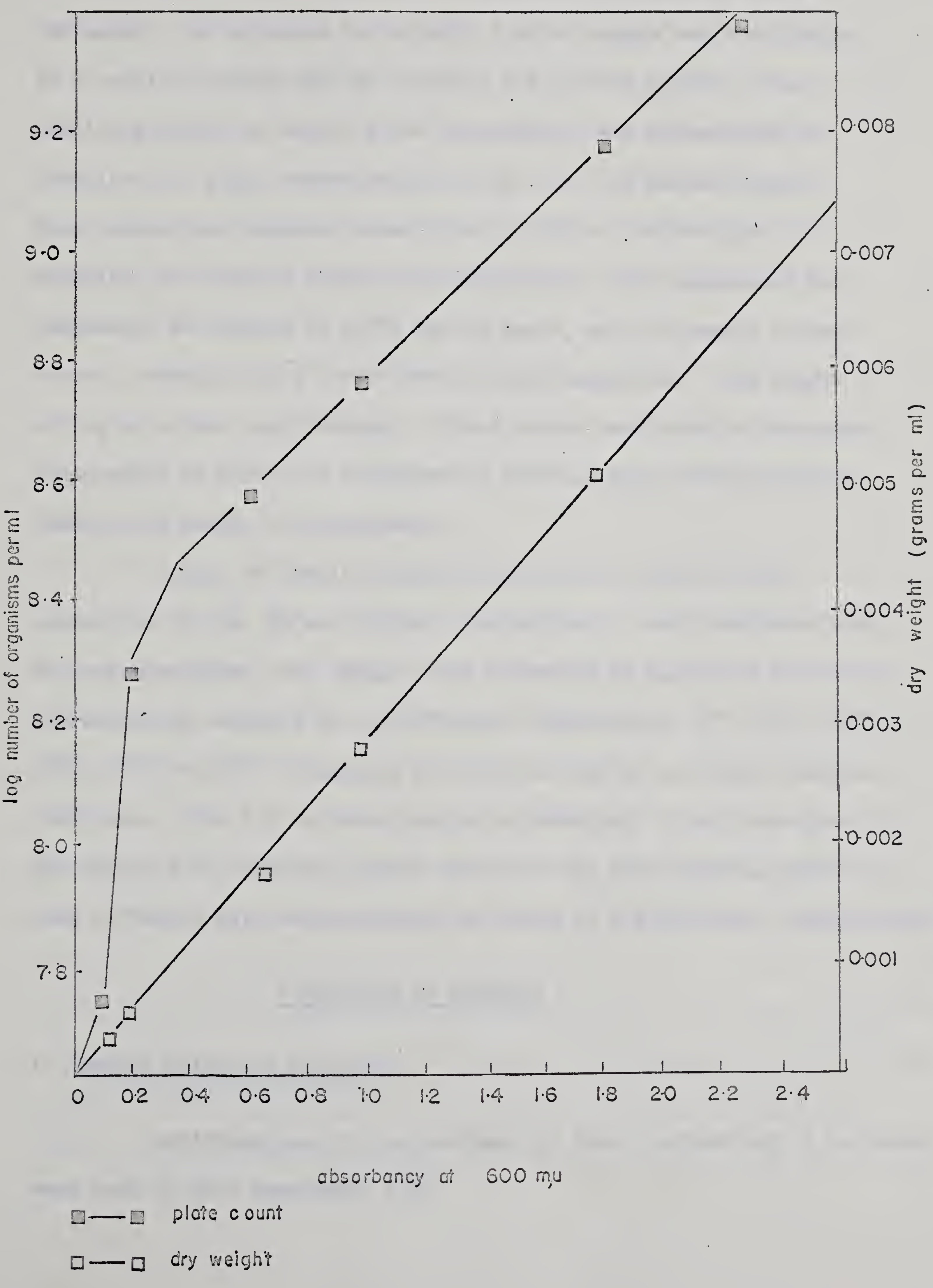
Cultures of S. aureus strain Cas 243, A.T.C.C. 14458, freeze dried onto porcelain beads, were grown out in double strength brain heart infusion broth (Baltimore Biological Laboratories) at 37°C for 24 hours. This strain is known to produce enterotoxin B (104). The stock cultures were transferred to meat infusion agar slants. After growth at 37°C for 24 hours the slant tubes were sealed and kept at 4°C. Subculturing took place every three months in order to retain the initial virulence of the organism.

STANDARD GROWTH CURVES

Many experimental techniques depend on some measure of bacterial growth. This may be the total mass of bacterial protoplasm/ml of culture, estimated directly as dry weight or indirectly as the light-scattering power of the culture. In some experiments it is more important to know the number of viable organisms and this is obtained by standard plate count methods.

A standard curve was prepared of dry weight v. optical density at 600 mμ.v. number of organisms/ml for S. aureus Cas 243. Fig. I.Double strength brain heart infusion broth was inoculated with S. aureus and incubated at 37°C. Samples in triplicate were taken at zero hour, and every subsequent hour for 24 hours. The optical density (O.D.) of each sample was measured on a Bausch and Lomb Spectronic 20. Dilutions of the sample were taken, and plated on brain heart infusion agar. After

FIG. I. STANDARD CURVE RELATING OPTICAL DENSITY, DRY WEIGHT AND NUMBER OF ORGANISMS PER ML OF <u>S. aureus</u> Cas 243 IN BROTH CULTURE.



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incubation at 37°C for 24 hours, the number of colonies per ml was estimated. To calculate dry weight, 3 ml of sample was centrifuged in a sterile weighed tube at 10,000 r.p.m. for 15 minutes. The resulting pellet of cells, after decantation, was resuspended in formalin at a final concentration of 1% v./v. and recentrifuged. This process was repeated using first, 0.85% w/v. saline plus 1% formalin, and finally sterile distilled water. The suspension was evaporated to dryness at 105°C for 24 hours, and the sample allowed to cool overnight in a desiccator to room temperature. The weight of the cells was then obtained. These curves were used in subsequent experiments to assist in interpreting relationships between optical density and number of organisms/ml.

Flasks of double strength brain heart infusion broth containing 1%, 4%, 8% and 12% NaCl respectively, were inoculated with the test organism. The samples were incubated in duplicate with their corresponding controls at six different temperatures, 4°C, 15°C, 20°C, 25°C, 30°C and 35°C. Sampling at zero time and at six hour intervals took place. The O.D. of each sample was obtained. From these data it was possible to construct growth curves of the test organism grown in four different salt concentrations of broth at six different temperatures.

PRODUCTION OF EXOTOXIN

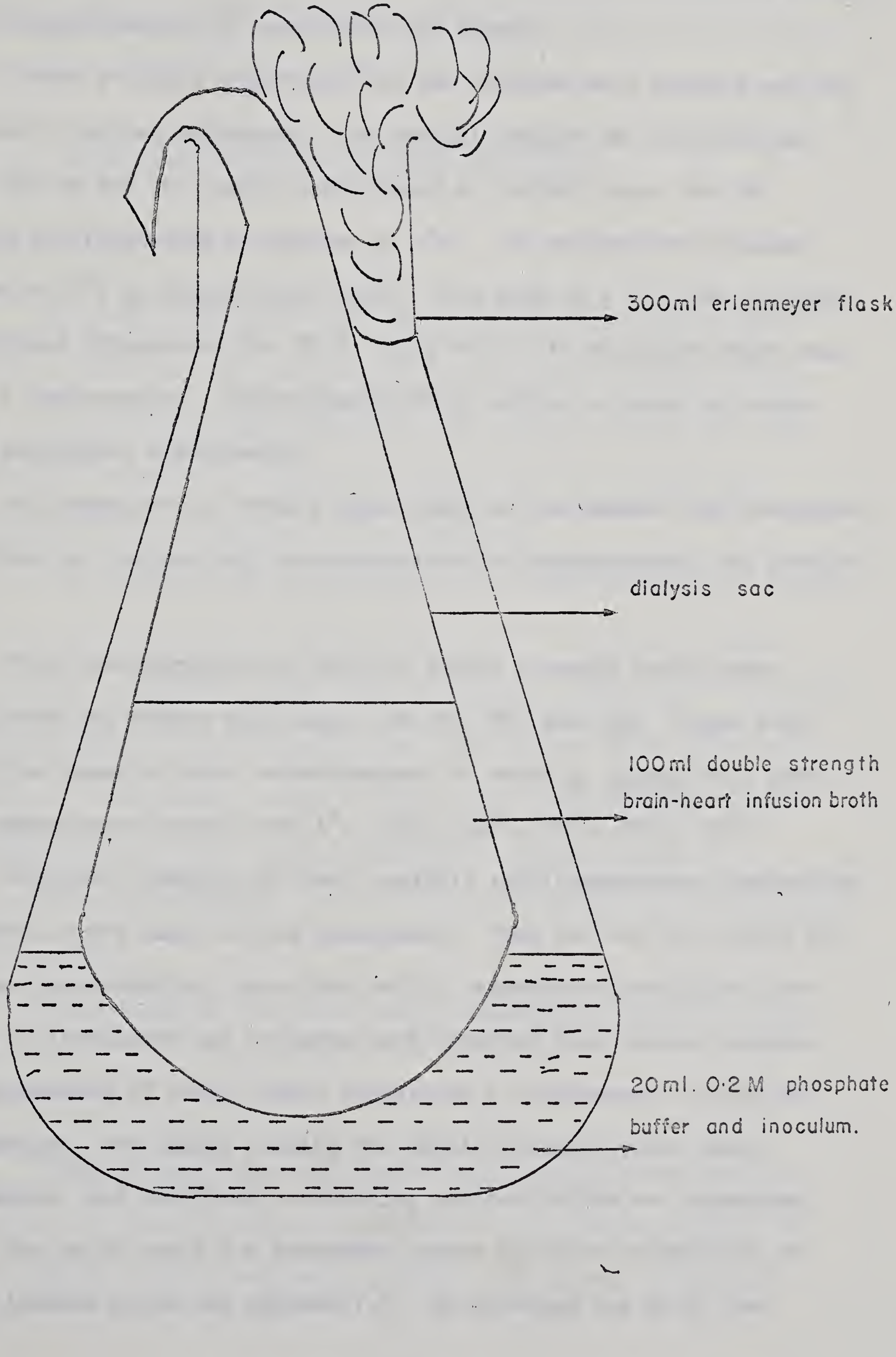
I. Method using sac cultures.

Modifications of a method used to detect enterotoxin B in cheese were used in this experiment (98).

S. aureus - Cas 243, was inoculated into sterile double strength brain heart infusion broth and incubated at 37°C for approximately 12 hours, or until the absorbancy of the suspension of organisms in broth reached approximately 0.44 at 600 mμ. This suspension was divided into 20 ml portions which were centrifuged at 10,000 r.p.m. for 15 minutes. The cells were resuspended in distilled water, centrifuged and finally suspended in 2 ml of sterile 0.2 M phosphate buffered distilled water ready for inoculation into sac cultures.

The sac culture assembly consisted of 100 ml of double strengh brain heart infusion broth in a closed dialysis bag suspended in 18 ml of 0.2 M phosphate buffered distilled water in a 300 ml Erlenmeyer flask. This is illustrated in Fig. II. The cellophane sacs were prepared from 1 7/8" diameter (3" flat width) dialysis tubing with an average porosity of 48 Å. (No. 4465-A2. Arthur Thomas Co. Philadelphia, U.S.A.) Seven-inch lengths were cut and soaked in distilled water. The tubing was tested for leaks after two knots had been tied at one end. The sac was inserted into the flask containing 18 ml of buffer. 100 ml of double strength brain heart infusion broth were then placed in the sac. A rubber band was used to secure the dialysis tubing to the top of the flask and a plug consisting of cotton wool covered with aluminum foil with holes punched through it, was used to close the whole assembly. The aluminum foil covering the cotton wool was found necessary, as frothing of the culture liquid occurred during incubation. The cotton wool Plugs, previously used, absorbed large amounts of the culture liquid. The entire sac culture assembly was autoclaved at 121°C for 15 minutes, and allowed to cool. Two ml

FIG. 11. SAC CULTURE ASSEMBLY USED FOR THE PRODUCTION OF CRUDE TOXIN.



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of buffer containing the inoculum was quickly added to the buffer outside the dialysis sac and the flask incubated in a temperature regulated rotary shaker at approximately 170 excursions per minute.

After set time intervals, the sac cultures were removed and the fluid outside the bag collected. The optical density of the fluid was taken at 600 mμ and the sample centrifuged at 10,000 r.p.m. for 20 minutes in a refrigerated centrifuge at 4°C. The supernatant obtained was stored at 4°C in sterile test tubes. One drop of a 1:10,000 solution of Merthiolate (Thimerosal No. 20.E. Lilly & Co.) in distilled water was added as a preservative. These samples were used as a source of crude toxin in subsequent experiments.

An experiment to obtain crude toxin by the method just described, from cultures at various salt concentrations and temperatures, was carried out.

Four concentrations of NaCl in double strength brain heart infusion broth and buffer were used:- 0%, 4%, 8%, and 12%. These were chosen as the range of salt concentrations in which _S. aureus_ will grow. The six temperatures chosen were 4°C, 15°C, 20°C, 25°C, 30°C, 35°C.

Duplicate samples for each possible salt/temperature combination, plus controls, were used in this experiment. Thus to test the effect of salt at one concentration, under one set of temperature conditions, for one week, 14 inoculated sac cultures were required plus eleven controls. Controls consisted of seven flasks containing all components except the micro-organisms, two flasks lacking the double strength brain heart infusion broth, and two flasks containing neither buffer nor organisms.

The pH of the 0.2 M phosphate buffer solution on addition of NaCl, was lowered below the optimum 7.2. By altering the pH of the

initial phosphate buffer, before NaCl addition, the error was eliminated. A greater buffering capacity of the broth must account for the relatively small changes in pH on addition of NaCl.

TESTS FOR THE DETECTION OF ENTEROTOXIN B

I. Serological method.

The test used for detecting the presence of enterotoxin B was a modification of the Oudin method of detecting antigens (105).

The principle of serological gel diffusion tests has been referred to in Chapter I. Using this principle the following is basically what was attempted in this experiment.

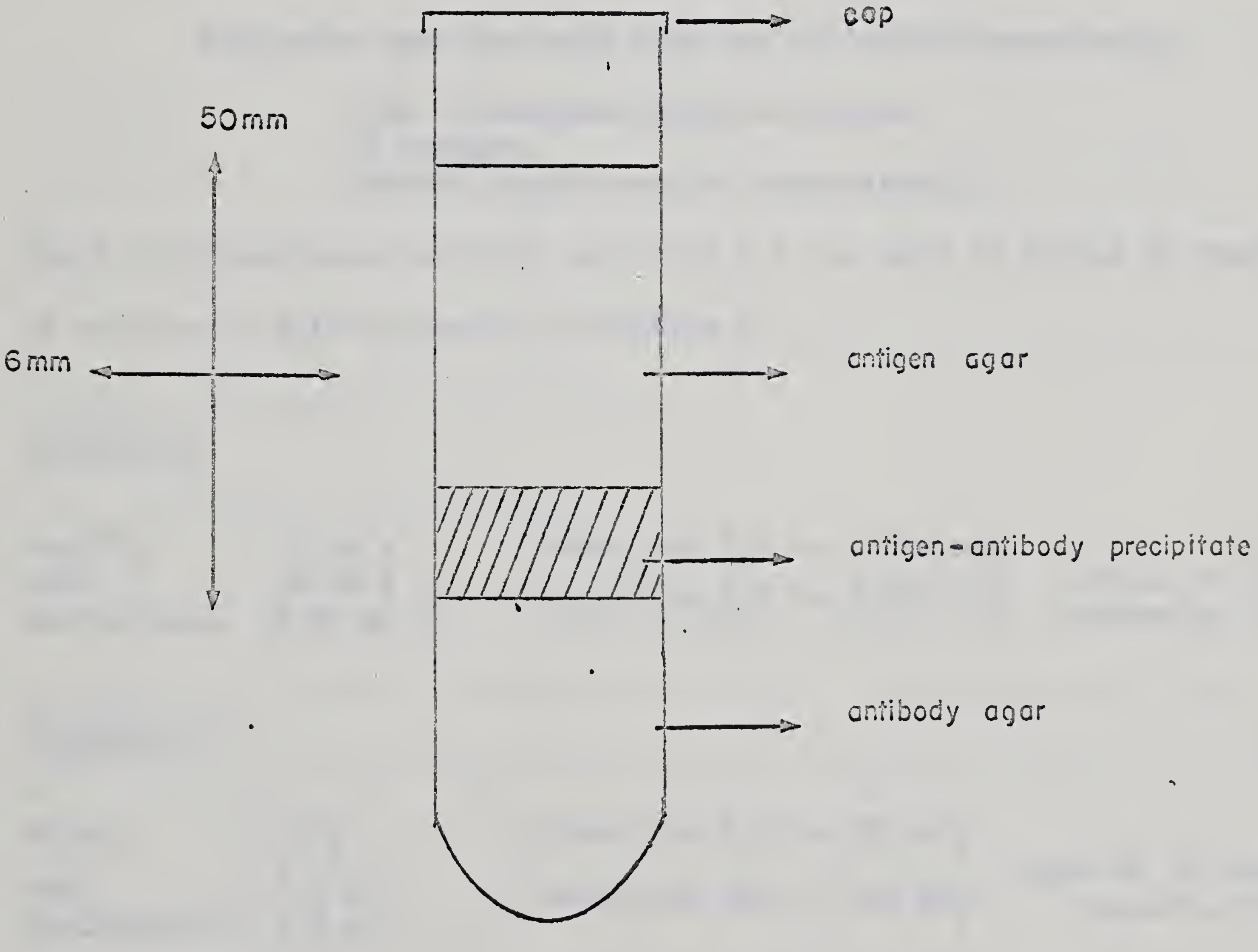
Gel diffusion tubes containing antiserum specific for enterotoxin B in soft agar, were overlaid with known or unknown antigens (Enterotoxin B) samples. Fig. III. The length of each zone of precipitation was measured after all tubes had been incubated at 30°C for 24 to 48 hours. A reference curve of known toxin concentration versus band length was plotted on semi-log paper. Values of unknown samples assayed concurrently were then taken directly from the standard reference curve.

The following is a more detailed account of this procedure:

(i) Preparation of assay tubes

'Kimax' tubes 6mm in diameter and 50 mm in length (Fisher Scientific Co.) were internally coated with 0.5% Ionagar (Oxoid), to prevent leakage at the glass-agar interface. This was achieved by

FIG. III. SINGLE DIFFUSION TUBE METHOD FOR DETERMINATION OF ENTEROTOXIN



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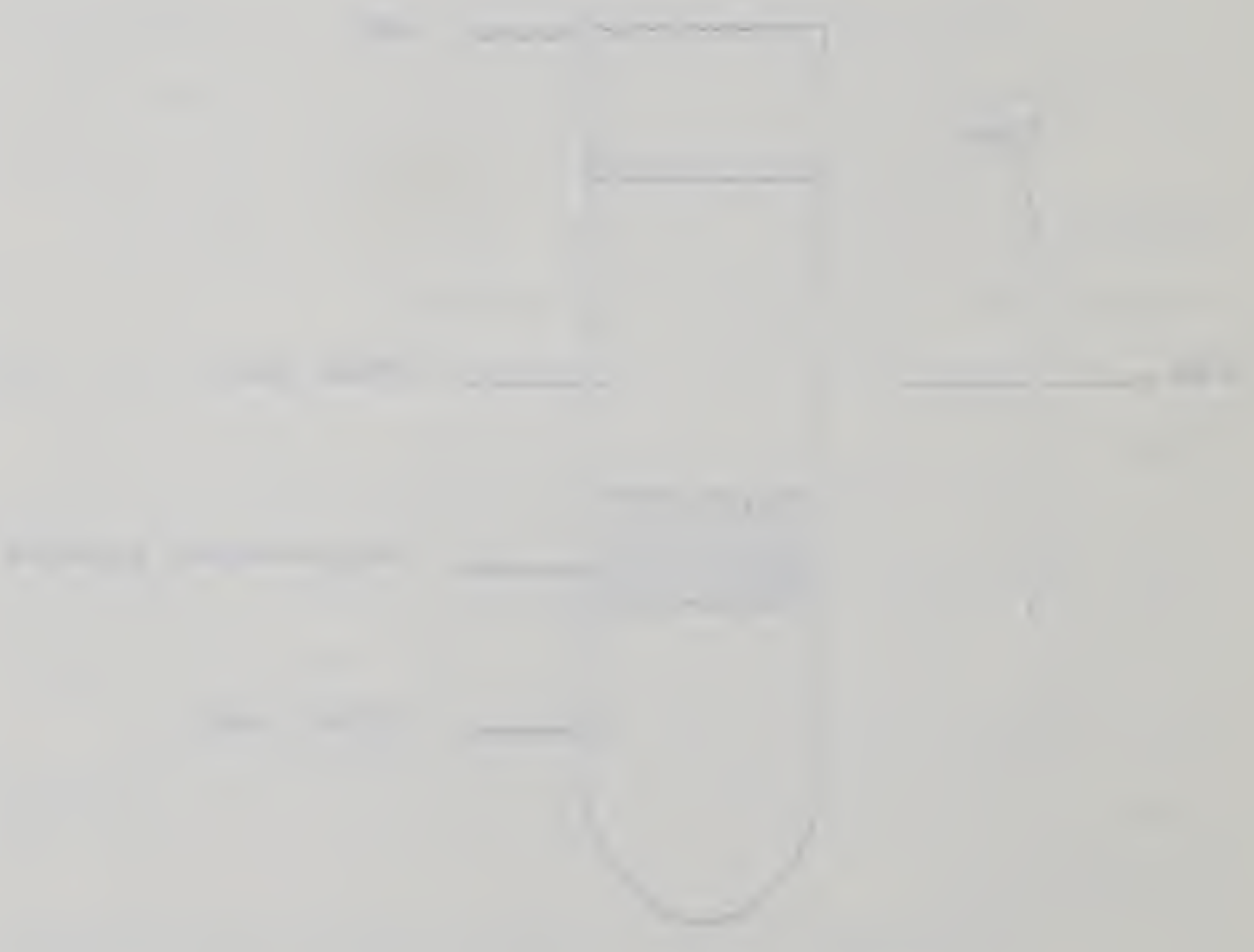


pipetting agar into and out of the tubes followed by vacuum drying over a desiccant. Foam plastic blocks provided convenient holders for the tubes and plastic labelling tape was used to identify tube locations. The tubes after vacuum drying, were half-filled with approximately 0.35 ml of antiserum agar.

(ii) Preparation of antiserum agar.

Antiserum agar was made from the following ingredients:

0.02 M Phosphate-buffered saline,
1% Ionagar,
Specific antiserum for enterotoxin B.

The 0.02 M phosphate-buffered saline pH 7.4 was made by mixing 85 parts of solution A with 15 parts of solution B.

Solution A:

Na_2HPO_4	11.36 g	+ distilled H_2O to 1,200 ml)	4,000 ml of Solution A stored at 5°C
NaCl	34.00 g)	+ distilled H_2O to 2,800 ml)	
Merthiolate	0.40 g)		

Solution B:

KH_2PO_4	2.7g	+ distilled H_2O to 300 ml)	1,000 ml of Solution B stored at 5°C
NaCl	8.5 g)	+ distilled H_2O to 700 ml)	
Merthiolate	1.0 g)		

1% Ionagar was prepared as follows:

Ionagar (Oxoid)	5 g)	1% Ionagar
0.02 phosphate buffered saline	495 ml)	

This mixture was heated for 10 min. at 121°C and filtered (Whatman No. 1). Filtration to clearness was accomplished by holding at 55°C during filtration.

(iii) Antiserum

Specific antiserum was obtained from Dr. R. B. Read at the Robert A. Taft Sanitary Engineering Centre, Cincinatti.

To 9 ml of phosphate buffered saline, at 48°C, was added 0.5 ml of antiserum. After thorough mixing 10 ml of 1% clarified agar, cooled to 48°C, was added to give a 1:40 final dilution in antiserum agar.

Warm Pasteur pipettes were used to fill the assay tubes and the agar was allowed to cool and set.

(iv) Application of test antigen.

An equal volume of crude toxin or control was added to the antiserum agar with a sterile Pasteur pipette. The tubes were sealed with Parafilm "M" (Marathon, Wisconsin) and incubated at 30°C.

Controls used:

No toxin : no serum
Toxin : no serum
No toxin : serum
Merthiolate : serum
Sterile broth : serum
Toxin, heated 30 min. at 100°C : serum

(v) Diffusion band measurements.

Routine measurements after incubation for 24 hr. and 48 hr. were made of the width of the zone produced by enterotoxin migration into the antiserum agar. Measurements were taken using a set of Vernier calipers (Fisher). All samples of crude toxin obtained were tested in duplicate by this method.

Four standard curves were prepared using highly purified, lyophilised enterotoxin B, kindly supplied by Dr. R. B. Read, Cincinatti. The toxin was rehydrated using four different salt concentrations in broth. This was to counteract any abnormalities in zone-development in gel-diffusion tests observed by some workers on addition of salts to the antigen sample (106). The standard curves are shown in Fig. IV.

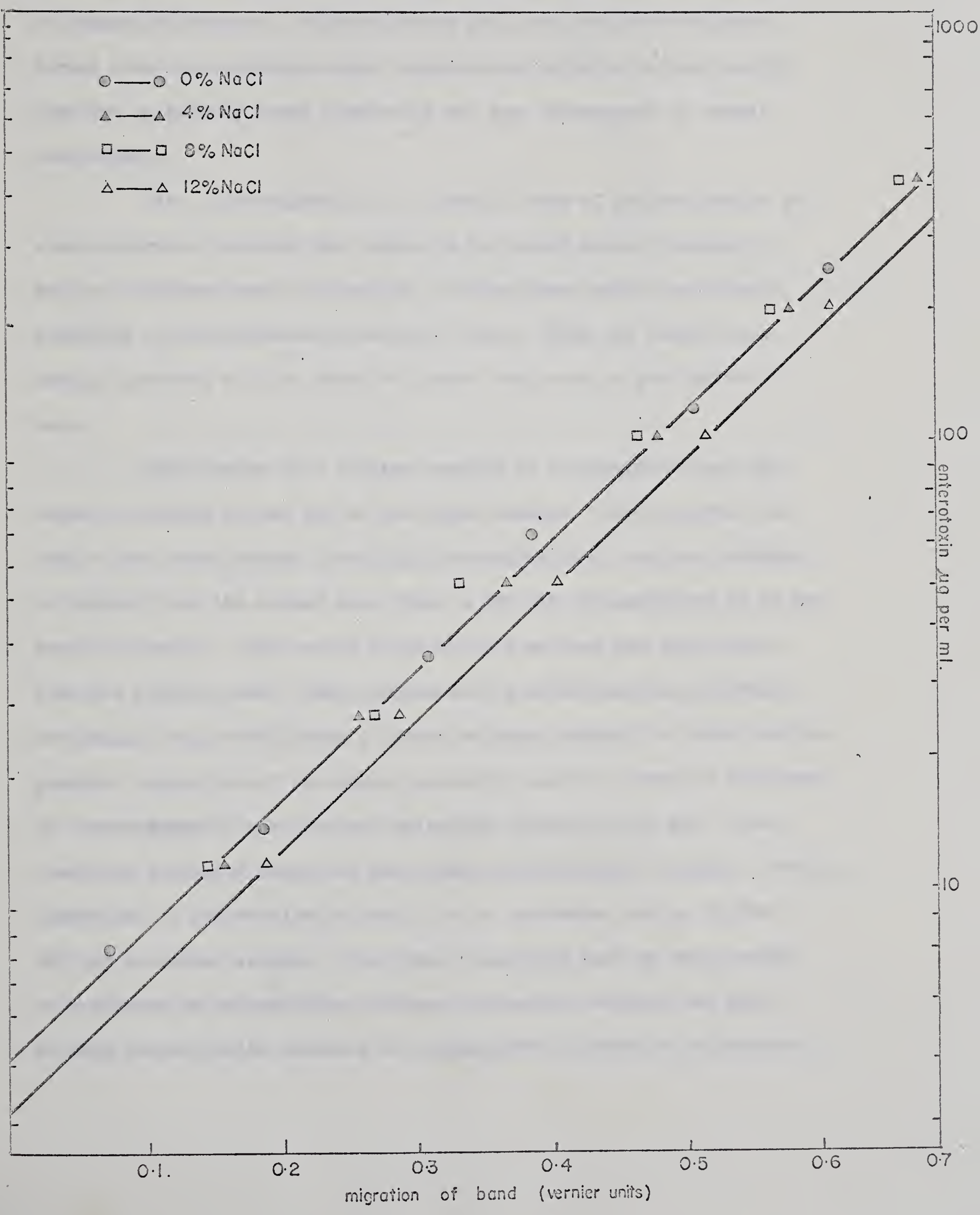
II. Electrophoretic Method

(i) Introduction

Zone electrophoresis provides a method of separating ionic mixtures and is based on differences among the electrophoretic mobilities of the constituent ions. A gel matrix is a latticed structure with pores of molecular dimension that can impose an appreciable frictional resistance to the passage of ions, provided the size of the pores approaches the dimensions of the migrating ions. With polyacrylamide gels the average pore size approaches the range of dimensions of proteins. The various protein fractions will therefore be differentially retarded to degrees proportional to their dimensions. Thus the separation of

FIG. IV. STANDARD CURVE FOR DETERMINATION OF ENTEROTOXIN B

(24 hour reading)



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mixtures is achieved in such a gel matrix through dimensional as well as charge differences. Polyacrylamide gels are synthetic polymers formed from low molecular weight chemicals obtainable in high purity. They are relatively inert chemically and are transparent at normal wavelengths.

Disc electrophoresis is a special form of polyacrylamide gel electrophoresis in which the sample to be tested passes through two gels of different pore size and pH. In the first gel, ions migrate according to electrophoretic mobility alone. That is, faster ions such as proteins will be ahead of slower ions such as weak acids or bases.

Application of a voltage results in a segregation and subsequent stacking of one set of ions upon another. Shortly after the sample ions have reached fixed high concentrations, they are arranged to migrate into the second gel, where a new set of conditions of pH and pore size exist. The ions of the weak acid or base now move faster than the protein ions. They continuously overtake and pass through the sample ions establishing a linear voltage gradient in which electrophoretic separation of the sample proceeds under the combined influence of electrophoretic mobility and molecular sieving by the gel. The resulting separated bands can range down to 50 microns in width. Best separation in polyacrylamide gels is with substances having 30,000 - 300,000 molecular weights. Very small molecules such as amino acids or a mixture of polypeptides, although separated, migrate too fast. As most extracellular products of staphylococci appear to be proteins

or enzymes, having moledcular weights within the optimum range for separation by disc gel electrophoresis, the possibility of using this method to detect their presence in the crude toxin preparations from the initial experiment was examined.

(ii) Equipment.

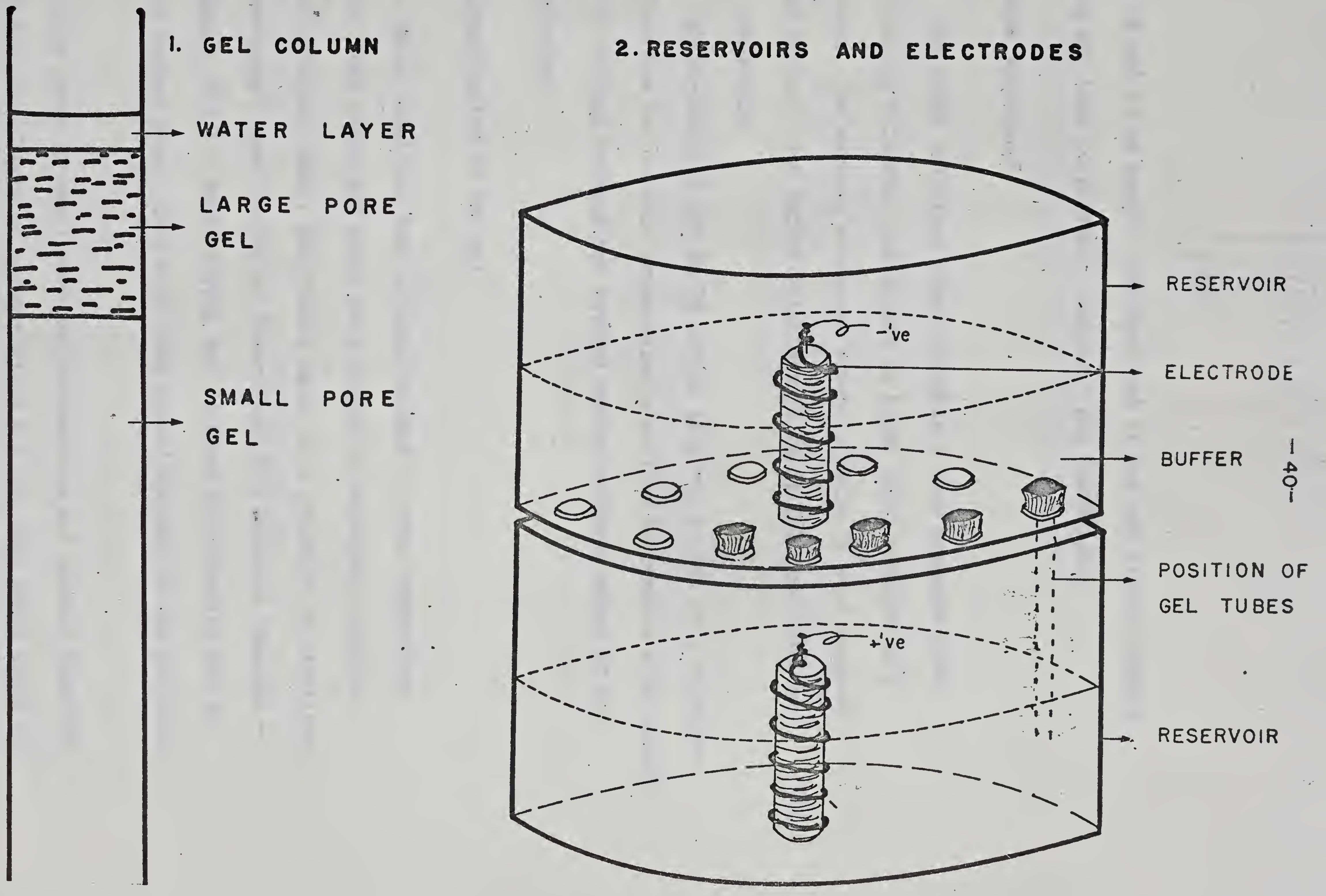
The apparatus used in this trial was constructed in the machine shop at the University of Alberta, and the method used was similar to that of other workers (107). The apparatus is illustrated in Fig. V.

The equipment consisted of two buffer reservoirs, constructed from inert, polystyrene, 7" in diameter and 4¼" in height. The upper buffer reservoir had twelve holes of 3/4" diameter in the bottom. Rubber bungs were fitted into these holes each with a 5/16" bore. The lower reservoir, an undrilled dish of similar dimensions, supported the upper reservoir. Number 30 platinum wire, wound around long axes of rigid polystyrene projecting from the base of the reservoirs, acted as the electrodes in the apparatus. The power supply was a Buchler constant current D.C. assembly.

Twelve glass tubes, 10" in length, o.d. 7 mm and i.d. 5 mm, were made from one piece of glass tubing. These tubes were used to support the gels.

A stand to support, and keep the gel tubes vertical during the first stages of the experiment was constructed from a block of foam plastic. Twelve B-D Vacutainer stoppers were fixed onto this block,

FIG. V. **DISC GEL ELECTROPHORESIS APPARATUS**



open end up and 10 mm apart. The open end of the cap fitted tightly around the gel tube to prevent leakage of gel ingredients.

(iii) Stock solutions.

The stock solutions shown in Table I were prepared with distilled water, filtered, and stored in brown glass bottles in a refrigerator. The working solutions shown in Table II were prepared on the day of use. The buffer solutions in Table III were used to fill the reservoirs.

Amido-Schwartz dye in 7% acetic acid was chosen as a fixative-stain. This was due to this preparation's ability to combine with protein material in the gel columns for several months without fading of the discs occurring.

(iv) Polymerization of the gel.

Stock solutions were allowed to warm to room temperature before use. The glass gel tubes were washed in detergent solution, rinsed in distilled water, and rinsed again in a solution of distilled water containing 1 part in 200 of Kodak Photo Flo Solution (Eastman Kodak Company, N.Y.). Rapid drying was achieved by attaching the gel tubes to a vacuum line. They were then placed upright in the gel tube stand.

The lower or small pore gel was made up and quickly pipetted into each tube to a depth of approximately 6.5 cm, care being taken to avoid the trapping of air bubbles. This was achieved with the use of a

TABLE I

Stock Solutions Used for Gel Electrophoresis

Solution A		Solution B	
IN HCl	48 ml	IN HCl	48 ml
TRIS*	36.6 gm	TRIS*	5.98 gm
TEMED*	0.23 ml	TEMED*	0.46 ml
Water to 100 ml (pH 8.9)		Water to 100 ml (pH 6.7)	
Solution C		Solution D	
Acrylamide*	28 gm	Acrylamide*	10 gm
BIS*	0.735 gm	BIS*	2.5 gm
Water to 100 ml		Water to 100 ml	
Solution E		Solution F	
Riboflavin*	4 mg	Sucrose	40 gm
Water to 100 ml		Water to 100 ml	

* TRIS = tris (hydroxymethyl) amino methane (Fisher Scientific Co.)

* TEMED = N,N,N^1,N^1 - tetramethylethylenediamine (Eastman Org. Chem. Co.)

* Acrylamide = $CH_2CHCONH_2$ (Eastman Org. Chem. Co.)

* BIS = N,N^1 - Methylenebisacrylamide (Eastman Org. Chem. Co.)

* Riboflavin - (acting as a catalyst with solution B) (Eastman Org. Chem. Co.)

TABLE II

Working solutions used for gel electrophoresis

A. Small pore gel

Solution 1

A	10 ml
C	20 ml
H_2O	10 ml

Solution 2

Ammonium persulphate 0.14 gm

H_2O to 100 ml

(Acting as a catalyst with Solution A)

40 ml of ①
+
40 ml of ②
= Small pore solution
pH 8.9

B. Large Pore gel

B	2 ml
D	4 ml
E	2 ml
F	8 ml

= large pore solution
pH 6.7

TABLE III

Buffer solution for reservoirs in gel electrophoresis

TRIS*	6.0 gm	= pH 8.3
Glycine	28.8 gm	
Water to 1000 ml		

1/10 of the strength of this stock buffer is used to fill the reservoirs.

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* TRIS = tris (hydroxymethyl) amino methane (Fisher Scientific Co.)

10 ml syringe fitted with a three inch needle. Deionised water was layered on top of the gel solution to a depth of five mm. The water settled on top of the denser gel solution and prevented the formation of a meniscus in the gel. Following water layering the tubes were placed directly under a daylight fluorescent bulb, the bulb being approximately three inches above the tips of the gels. Photopolymerization of the gel solution proceeded for about 45 mins., following which the water layer was removed by absorption with a piece of filter paper. A large pore spacer gel was prepared and placed by means of a syringe on top of the small pore gel to a depth of 2½ cm. This gel was water layered, as before, and the tubes placed under the lamp for photopolymerization. After approximately 30 minutes the water layer was absorbed and the gel tubes removed from the gel stand.

(v) Application of a current.

Following photopolymerization, the tubes, large pore gel uppermost, were inserted into the rubber bungs of the upper reservoir. A hanging drop of buffer was placed on the bottom of each gel tube and the upper reservoir lowered onto the bottom reservoir, which contained 400 ml of chilled 1/10 strength stock buffer solution. The bottom reservoir was connected to the anode.

Samples of crude toxin and the various controls to be analysed, were added to the well at the top of the gel tubes by means of a tuberculin syringe. Each 0.1 ml of sample, was overlaid with 0.2 ml of 25% sucrose solution to prevent loss of the sample during the run. The

The upper reservoir was connected to the cathode and gently filled with chilled 1/10 stock buffer solution by siphoning. The buffer solution contained 1 ml of 0.001% Brom-phenol Blue as a marker dye.

The current was regulated to 2.5 amps/tube, thus giving the appropriate current density. Electrophoresis lasted approximately 60 minutes, or until the marker dye had moved within 2 cm of the end of the gel tube. The temperature of the buffer solution rose no more than 2°C throughout the experiment.

(vi) Staining and destaining of gels.

At the completion of electrophoresis the gel columns were loosened and removed from their tubes by gently "rimming" them at their lower ends with a long, blunted, hypodermic needle through which water was flowing.

Each gel was immediately immersed in 5 ml of fixative-stain solution for approximately 80 mins. At the end of this period, the fixative-stain solution was decanted and the gels rinsed in tap water.

Destaining by frequent changing of a 7% acetic acid solution, in which the gels were immersed over a period of 3 days, produced good results.

(vii) Recording of results.

The gels were placed in stoppered glass tubes 10 cm in length 0.9 mm o.d., 0.6 mm i.d. filled with 7% acetic acid. The bands in the

gels were recorded densitometrically using a disc gel attachment on a Densicord 542 densitometer (Photovolt Corp., New York).

During electrophoresis it was difficult to arrange that each sample ran to exactly the same length, hence the chart speed of the densitometer was adjusted so that the trace obtained for each gel was of a standard length. The peaks on each trace which corresponded to visible bands in the appropriate gel were marked with numbers. The gels were then photographed against a diffuse white background, illuminated from the rear. When the corresponding trace and photograph were in juxtaposition each visible band on the photograph could be related to a peak on the trace. Even so, some of the very fine and faint bands in the gels did not show well on either the densitometric traces or photographs. Therefore, a diagrammatic interpretation was made for each gel, taking account of all 3 types of observation, i.e., the visual, the photographic and the densitometric.

(viii) Identification of zones in gels.

In order to estimate the sensitivity of the test, as a means of identifying enterotoxin and other extracellular proteins and enzymes, it was decided to elute and attempt to identify each band from the gel. This was achieved in the following manner:

Unstained gels, taken directly from the gel tubes after electrophoresis, were suspended for ten minutes or longer in liquid nitrogen at -60°C, care being taken that they remained vertical throughout freezing. The gels in a frozen state were then placed between two pieces of plate

glass, the edges of which had been measured off in millimetres. Using a sharp razor blade the gels were sliced in half lengthways. One half was placed in stain-fixative solution for half an hour and then destained in 7% acetic acid, the other half was sliced into sections each approximately 2 mm in length. Each gel slice was then placed in a small spectrophotometer tube with 0.5 ml of buffer. Two buffers were used in this elution technique. A 0.1M acetate buffer, pH 5.5, and a 0.2M Veronal buffer at pH 7.6. Using these two buffers it was found possible to elute several of the components from the gel.

Sliced gel samples were stored overnight at -10°C. They were allowed to thaw slowly and were homogenised using a Teflon pestle tissue grinder. The liquid was decanted, the broken pieces of gel remaining in the bottom of the sample tube. It seemed necessary to concentrate the sample in order to increase the sensitivity of the various tests. Therefore each eluate was dialysed against 0.1M. TRIS + Ca + Mg - buffer overnight at 4°C and evaporated to dryness. This dried material was used throughout the following experiments.

(a) Protein

The protein content of the unstained, dried, sliced gels was examined using the 'Lowry' method (108) as follows:

Reagents:

A. 2% Na_2CO_3 in 0.1N NaOH
B. 0.5% $CuSO_4$ $5H_2O$ in 1% potassium tartrate
C. Alkaline copper solution.
50 mls of A + 1 ml B
D. Folin Phenol reagent diluted 1 part to 2 parts H_2O.

Method

To 0.5 ml of Veronal buffer plus sample, 2.5 ml of reagent C was added. This was mixed and allowed to stand for ten minutes at room temperature before 0.25 ml of reagent D was added and quickly mixed. After letting this mixture stand for at least 30 minutes the optical density of each sample was read at 500 mμ. A blank using 0.5 ml of buffer was used in place of the sample. Using serum-albumin, a standard curve was constructed by the same method. This is illustrated in Fig. VI.

(b) Enterotoxin

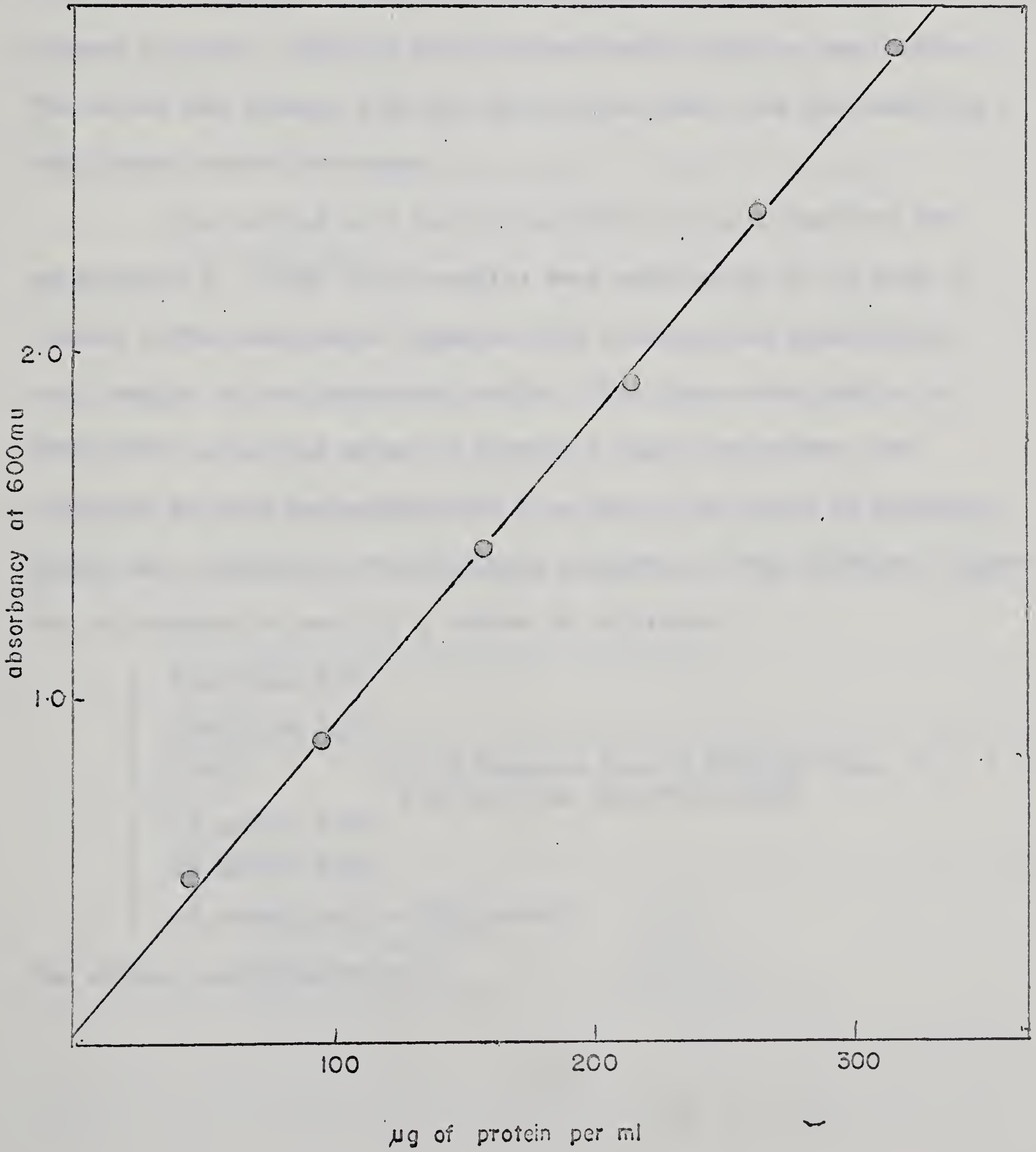
For controls in this experiment various dilutions of pure toxin were passed through the disc electrophoresis apparatus and the resulting gels sliced. As well as being able to identify the enterotoxin band in the crude sample, this experiment was also designed to estimate the sensitivity of the disc electrophoresis technique, by comparing the visibility of band produced at various concentrations of pure toxin.

The test used to identify the band location of enterotoxin in the gels was a modification of the Ouchterlony plate technique (109).

Agar used in this technique was composed of the following ingredients:

Oxoid Ionagar No. 2	1.2%	made up to 100 ml with deionized water
Sodium azide	1%	
E.D.T.A.	30 mg	

FIG. VI. STANDARD CURVE RELATING OPTICAL DENSITY AND PROTEIN CONCENTRATION



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This material was sterilised at 10 lb. p.s.i. for 10 min. Glass slides 3" by 2", which had been washed in detergent and rinsed in water, ethanol and distilled water respectively, were dried, warmed and dipped into molten agar. The slides were placed in an almost vertical position until excess agar had drained away, leaving a thin layer. Six ml of agar were then pipetted onto the slide and allowed to cool. Wells in the agar were made using an agar cutter. The cutter was pressed into the gel, lifted away, and the resulting agar plugs removed by vacuum.

The central well was filled with antiserum specific for enterotoxin B. Crude toxin samples were rehydrated in one drop of Veronal buffer and placed together with controls and sensitivity test samples in the peripheral wells. The slides were kept in a desiccator containing water to provide a humid atmosphere, and incubated at room temperature for five days. The bands of precipitation were stained by the following procedure. Each slide was dipped for ten minutes in each of a series of solutions:

Distilled H_2O
Distilled H_2O
Stain (0.1% Thiazine Red, R.(Allied Chem. Co. New York) in 1% acetic acid)
1% acetic acid
1% acetic acid
1% acetic acid + 1% glycerol

The slides were dried at 37°C.

(c) Hemolysins

The sliced gel samples, which had been concentrated, were put into individual sterile antibiotic assay cups on rabbit blood agar plates. A small drop of Veronal buffer was added to each cup. The plates were incubated at 37°C for 48 hours and then examined for α hemolysis.

The samples from a second gel were similarly treated by placing them in sterile assay cups on sheep's blood agar plus one drop of acetate buffer. Incubation at 37°C for 48 hours was followed by incubation at 4°C for 24 hours. The plates were examined for β hemolysis.

(d) Coagulase

The presence of coagulase was determined by incubating 0.1 ml of sample for one hour in 0.9 ml Difco coagulase plasma at 37°C (110).

Samples tested included broth cultures plus whole organisms, crude toxin preparations, and eluates from sliced gel columns.

(e) Deoxyribonuclease and Phosphatase

Deoxyribonuclease and phosphatase were detected by a plate assay method (111).

Assay mixture:

$MgCl_2 - 6 H_2O$	0.004M
$CaCl_2$ (anhydrous)	0.004M
Noble agar	1.5% (w/v)
TRIS - HCl buffer	0.01M (pH 8.8)

The mixture was heated to melt the agar and cooled to 45°C. Deoxyribonucleic acid was added and the mixture poured into Petri dishes to

solidify. Concentrated samples from gels were added together with one drop of 0.2M Veronal buffer 7.2 pH to antibiotic assay cups previously embedded in the assay mixture. Plates were incubated at 37°C in a moist atmosphere overnight, after which the plates were developed as follows. The plates were first flooded with 10% cold trichloracetic acid for fifteen minutes, the excess acid then removed. Following this the plates were flooded with molybdicaminonapthol-sulfonic acid reagent prepared as follows:-

1 ml of molybdic acid reagent:

$(NH_4)_6\ Mo_7O_{24}\ 4\ H_2O$, 2.5%(w/v) in 3 NH_2SO_4

0.4 ml Amino Napthol sulfonic acid reagent:

1 - amino - 2 napthol - 4 - sulfonic acid, 0.25% w/v

$NaHSO_3$, 15% w/v
Na_2SO_3, 0.25% w/v

8.0 ml of distilled water

The plates were then incubated for 15 minutes at room temperature and examined for clearing, indicating depolymerase activity around the assay cups. Phosphatase activity was indicated by a marked blueing of the clear zones.

(f) Lipase

Lipase activity was detected by placing samples of sliced gels in assay cups on tributyrin agar for 48 hours at 37°C.

Tributyrin agar was made up as follows:

1% Tween 80	0.5 ml
Tributyrin	0.25 ml
Isotonic saline	4 ml

These were mixed and steamed for 15 minutes. The emulsion was mixed with 20 ml of nutrient agar and plates were poured. Evidence of lipase activity resulted in a clearing of the agar around the assay cups.

(g) Hyaluronidase

Hyaluronidase prevents a protein hyaluronic acid complex from forming a typical 'mucin clot' when acetic acid is added (112).

To 0.5 ml samples of sliced gels dissolved in Veronal buffer, was added 1 ml of hyaluronic acid reagent consisting of 10 parts 2% solution hyaluronic acid (Canlab) in distilled water + 10 parts rabbit serum diluted 1/10 in saline + 20 parts distilled water. The preparation was incubated for twenty minutes at 37°C. After cooling in ice, one drop of acetic acid was added and clot formation observed.

RESULTS

RESULTS

Growth in broth.

The growth and survival of the test organism S. aureus Cas 243 in broth at several salt concentration/temperature combinations, was measured as change in optical density of the broth with time. The results are given in Table IV. The results are expressed graphically as growth curves showing changes in absorbancy at 600 mμ with time of incubation of the cultures in hours (see Fig. VII). Uninoculated control broths showed negative growth and zero absorbancy readings.

It was observed that cells grown in media of high salt concentration grew more rapidly on transfer to fresh high salt media than cells which had been grown in salt free media.

Growth in sac cultures

Using the standard curve relating O.D. to number of organisms/ml (Fig. I, page 27.) it was found that the number of test organisms present in the buffer solution of the sac cultures after inoculation was approximately 300 x 10^6 orgs./ml. The growth of the organisms throughout subsequent incubation was followed by changes in absorbancy of the medium at 600 mμ. This is shown in Table V.

It was noted that the readings were comparable to those obtained from growth of the organism in broth. The time taken for the culture to reach the infinity absorbance reading being approximately the same. From this we may assume that growth in sac cultures and growth in broth

Table IV

Effects of time, temperature and salt concentration on the growth of S. aureus Cas 243 in broth culture as measured by changes in O.D.

Temp. (°C)	% NaCl	Time of incubation (days)								
		½	1	2	3	4	5	6	7	8
35°C	0	1.20	∞	∞	∞	∞	∞	∞	∞	∞
		1.40	∞	∞	∞	∞	∞	∞	∞	∞
	4	1.00	2.80	∞	∞	∞	∞	∞	∞	∞
		1.20	2.80	∞	∞	∞	∞	∞	∞	∞
	8	0.30	1.35	2.50	∞	∞	∞	∞	∞	∞
		0.20	1.60	2.80	∞	∞	∞	∞	∞	∞
	12	0.00	.03	.06	2.70	∞	∞	∞	∞	∞
		0.00	.05	.07	2.80	∞	∞	∞	∞	∞
30°C	0	1.90	∞	∞	∞	∞	∞	∞	∞	∞
		1.70	∞	∞	∞	∞	∞	∞	∞	∞
	4	1.30	2.80	∞	∞	∞	∞	∞	∞	∞
		1.40	2.50	∞	∞	∞	∞	∞	∞	∞
	8	0.00	.80	1.90	2.80	∞	∞	∞	∞	∞
		0.00	1.00	1.90	2.80	∞	∞	∞	∞	∞
	12	0.00	.03	.40	1.70	2.00	2.40	2.80	∞	∞
		0.00	.01	.20	1.50	2.00	2.30	2.70	∞	∞
25°C	0	0.20	2.00	∞	∞	∞	∞	∞	∞	∞
		0.10	2.00	∞	∞	∞	∞	∞	∞	∞
	4	0.01	1.90	2.80	∞	∞	∞	∞	∞	∞
		0.02	1.80	2.80	∞	∞	∞	∞	∞	∞
	8	0.00	.01	2.00	2.60	∞	∞	∞	∞	∞
		0.00	.03	2.00	2.70	∞	∞	∞	∞	∞
	12	0.00	.10	.10	.30	.80	1.70	1.90	2.00	2.00
		0.00	.10	.10	.30	.70	1.50	1.90	2.00	2.00
20°C	0	0.00	.80	∞	∞	∞	∞	∞	∞	∞
		0.00	.70	∞	∞	∞	∞	∞	∞	∞
	4	0.00	.20	1.20	∞	∞	∞	∞	∞	∞
		0.00	.25	1.30	∞	∞	∞	∞	∞	∞
	8	0.00	.01	.80	1.90	∞	∞	∞	∞	∞
		0.00	.00	.90	2.00	∞	∞	∞	∞	∞
	12	0.00	.01	.01	.02	.03	.05	.09	1.00	1.20
		0.00	.01	.01	.02	.035	.055	.09	1.00	1.30
15°C	0	0.00	0.00	0.00	0.00	0.00	0.30	0.90	∞	∞
		0.00	0.00	0.00	0.00	0.00	0.20	0.70	∞	∞
	4	0.00	0.00	0.00	0.00	0.00	0.10	0.80	2.80	∞
		0.00	0.00	0.00	0.00	0.00	0.20	0.90	2.90	∞
	8	0.00	0.00	0.00	0.00	0.00	0.00	0.20	1.00	1.80
		0.00	0.00	0.00	0.00	0.00	0.00	0.20	1.10	1.90
	12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40°C	0, 4, 8, 12%	NO GROWTH								

[illegible]

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

FIG. VII. EFFECT OF SALT CONCENTRATION AND TEMPERATURE ON THE GROWTH OF *S. aureus* Cas 243 IN BROTH CULTURE.

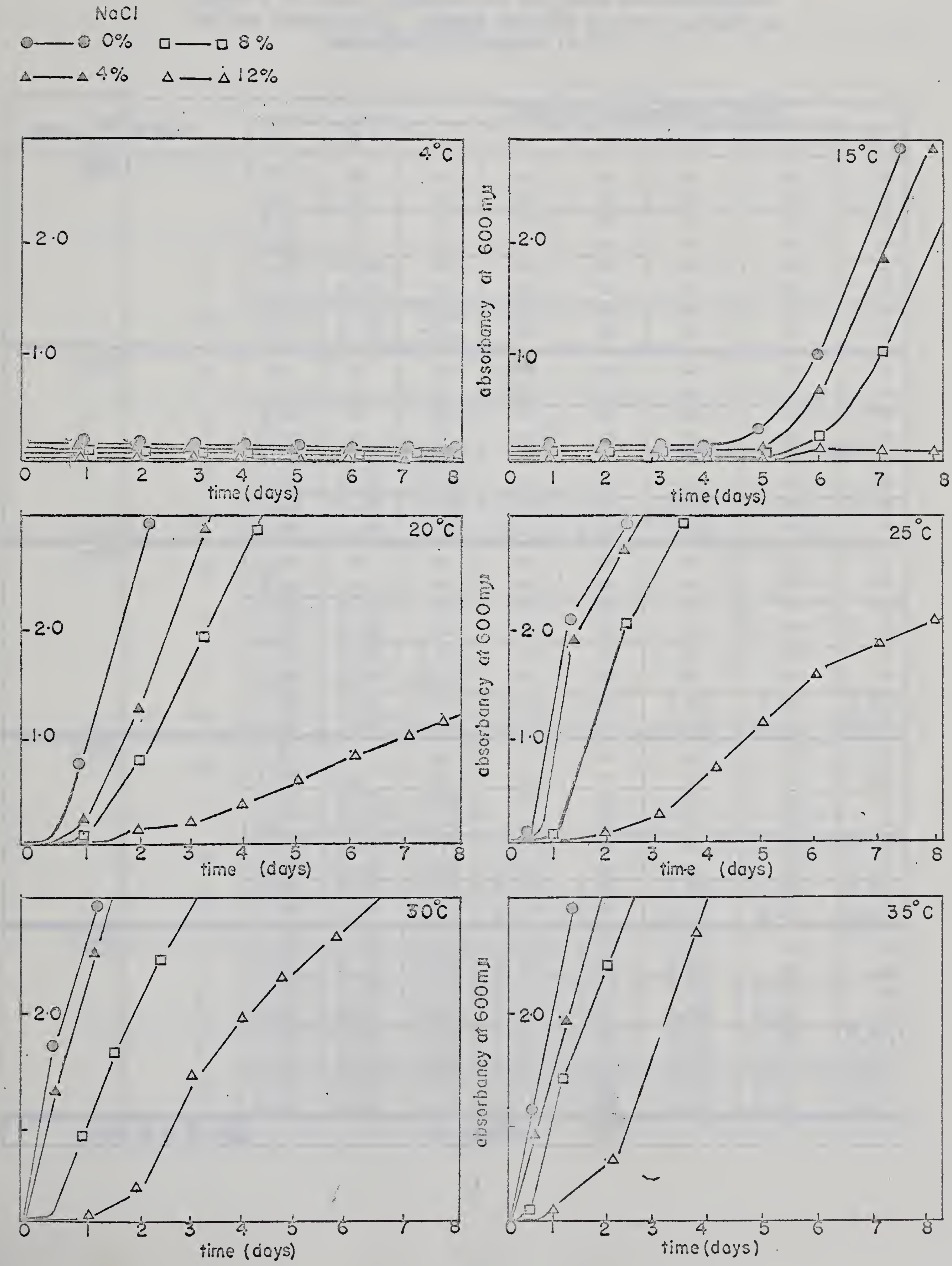

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Table V

Effects of time, temperature and salt concentration on the growth of S. aureus Cas 243 in sac culture as measured by changes in O.D.

Temp. °C	% NaCl	Time of incubation (days)							
		1	2	3	4	5	6	7	8
35°C	0	∞	∞	∞	∞	∞	∞	∞	∞
		∞	∞	∞	∞	∞	∞	∞	∞
	4	2.08	∞	∞	∞	∞	∞	∞	∞
		2.90	∞	∞	∞	∞	∞	∞	∞
	8	1.80	2.00	∞	∞	∞	∞	∞	∞
		1.90	2.20	∞	∞	∞	∞	∞	∞
	12	0.10	0.15	0.20	∞	∞	∞	∞	∞
		0.11	0.13	0.24	∞	∞	∞	∞	∞
30°C	0	∞	∞	∞	∞	∞	∞	∞	∞
		∞	∞	∞	∞	∞	∞	∞	∞
	4	2.00	∞	∞	∞	∞	∞	∞	∞
		2.00	∞	∞	∞	∞	∞	∞	∞
	8	0.52	1.30	2.00	∞	∞	∞	∞	∞
		0.80	1.50	2.50	∞	∞	∞	∞	∞
	12	0.12	0.27	0.35	1.70	2.20	2.50	∞	∞
		0.26	0.25	0.36	1.60	2.00	2.50	∞	∞
25°C	0	2.00	∞	∞	∞	∞	∞	∞	∞
		2.00	∞	∞	∞	∞	∞	∞	∞
	4	1.90	2.80	∞	∞	∞	∞	∞	∞
		2.00	2.06	∞	∞	∞	∞	∞	∞
	8	1.90	2.00	2.50	∞	∞	∞	∞	∞
		2.00	2.00	2.50	∞	∞	∞	∞	∞
	12	0.12	1.00	1.10	1.00	0.90	1.00	2.00	1.90
		0.10	1.00	1.10	1.10	1.10	1.20	2.20	2.00
20°C	0	1.60	∞	∞	∞	∞	∞	∞	∞
		1.80	∞	∞	∞	∞	∞	∞	∞
	4	1.30	1.80	1.99	∞	∞	∞	∞	∞
		1.75	1.80	2.40	∞	∞	∞	∞	∞
	8	0.80	1.25	1.75	2.00	∞	∞	∞	∞
		0.90	1.25	1.90	2.00	∞	∞	∞	∞
	12	0.90	0.80	1.00	1.06	1.50	1.75	1.90	2.00
		0.70	0.70	1.30	1.20	1.50	1.70	2.00	2.00
15°C	0	0.28	0.30	0.34	0.28	1.80	2.00	∞	∞
		0.28	0.30	0.28	0.28	2.00	2.20	∞	∞
	4	0.20	0.22	0.24	0.21	0.43	0.45	1.70	∞
		0.20	0.24	0.23	0.28	0.45	0.48	1.50	∞
	8	0.19	0.18	0.20	1.25	0.35	0.37	1.80	2.00
		0.10	0.15	0.23	1.22	0.31	0.38	1.50	2.00
	12	0.12	0.13	0.10	1.15	0.20	0.17	0.15	0.15
		0.16	0.10	0.12	1.15	0.10	0.16	0.15	0.12
4°C	0, 4, 8, 12%	NO GROWTH							

[illegible]

[illegible]
[illegible]
[illegible]

[illegible]

cultures of the test organism were similar. This means that all essential nutrients required by the organism for normal growth were able to pass through the dialysis bag from the broth to the buffer solution in the sac culture assembly.

Toxin Production

The capacity of the test organism to produce toxin under various conditions was expressed as the width of the migrating antigen-antibody precipitate band, measured in vernier units, after 24 hr. incubation of samples of antigen and antibody at 30°C. Results are shown in Table VI. The controls used in this experiment, and all samples grown in 12% salt or at 4°C, showed no precipitate band. Negative results were also obtained from the following salt/temperature combinations, 4% NaCl at 30°C, 25°C, 20°C, 15°C and 4°C. At 8% NaCl concentration, no toxin was produced either at 30°C, 25°C, 20°C, 15°C and 4°C. To estimate the amount of toxin/ml produced at the various salt concentrations the standard curve shown in Fig. IV was used. The information obtained was expressed graphically as the amount of toxin produced in µg/ml, with time in hr (see Fig. VIII). In order to compare the production of toxin vs time, with the growth of the organism vs time, Fig. IX was constructed on the same scale as Fig. VIII.

Disc-gel electrophoresis

Electrophoresis of the samples obtained from sac cultures, and their corresponding controls, revealed a maximum of nineteen stainable

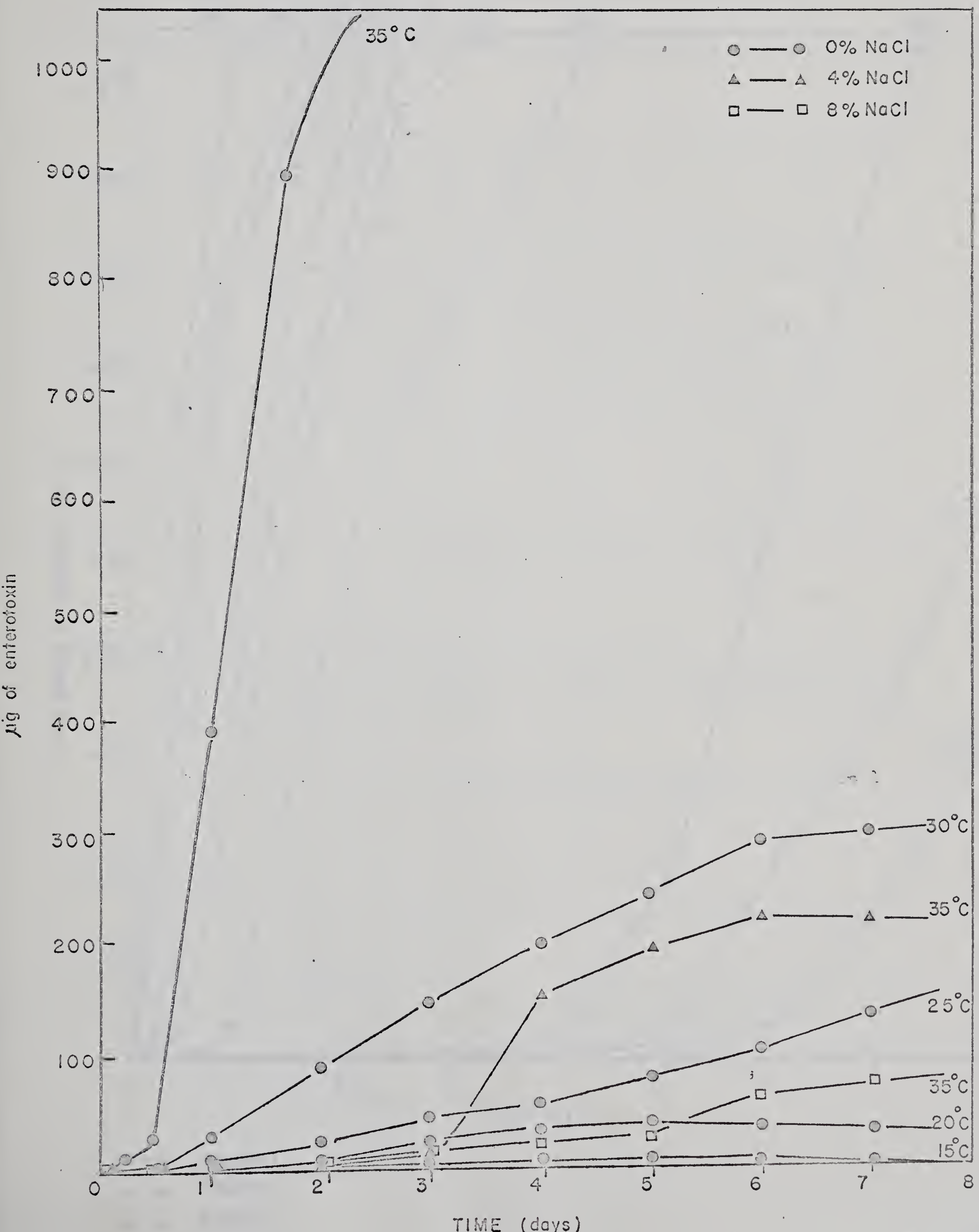
Table VI

Effect of temperature, time and salt concentration on the migration of precipitin bands (vernier units)

Age of sac culture sample (hr.)	Vernier units with 0% salt at incubation temperatures and times of diffusion tubes:							
	35°C		30°C		25°C		20°C	
	24	48	24	48	24	48	24	48
6	0.12	0.18	0.00	0.00	0.00	0.00	0.00	0.00
	0.13	0.19	0.00	0.00	0.00	0.00	0.00	0.00
24	0.76	1.09	0.22	0.24	0.18	0.20	0.00	0.00
	0.69	1.00	0.25	0.27	0.16	0.19	0.00	0.00
48	0.84	1.17	0.50	0.53	0.32	0.34	0.19	0.20
	0.82	1.15	0.48	0.51	0.34	0.37	0.12	0.21
72	0.85	1.19	0.59	0.60	0.40	0.48	0.31	0.33
	0.86	1.17	0.60	0.62	0.42	0.45	0.34	0.36
120	no reading		0.68	0.70	0.52	0.55	0.38	0.40
	no reading		0.66	0.68	0.50	0.53	0.40	0.42
168	no reading		0.68	0.70	0.58	0.60	0.31	0.33
	no reading		0.68	0.70	0.50	0.60	0.33	0.34

Age of sac culture sample (hr.)	Vernier readings with incubation of diffusion tubes at 35°C with salt concentration and incubation time (hr.) of:			
	4%		8%	
	24	48	24	48
6	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00
48	0.15	0.18	0.15	0.16
	0.16	0.19	0.13	0.14
72	0.21	0.23	0.21	0.22
	0.22	0.25	0.22	0.23
120	0.60	0.62	0.32	0.33
	0.62	0.65	0.31	0.32
168	0.60	0.62	0.45	0.48
	0.64	0.66	0.42	0.44

FIG. VIII. ENTEROTOXIN PRODUCTION IN SAC CULTURES OF 0%, 4% and 8% SODIUM CHLORIDE.



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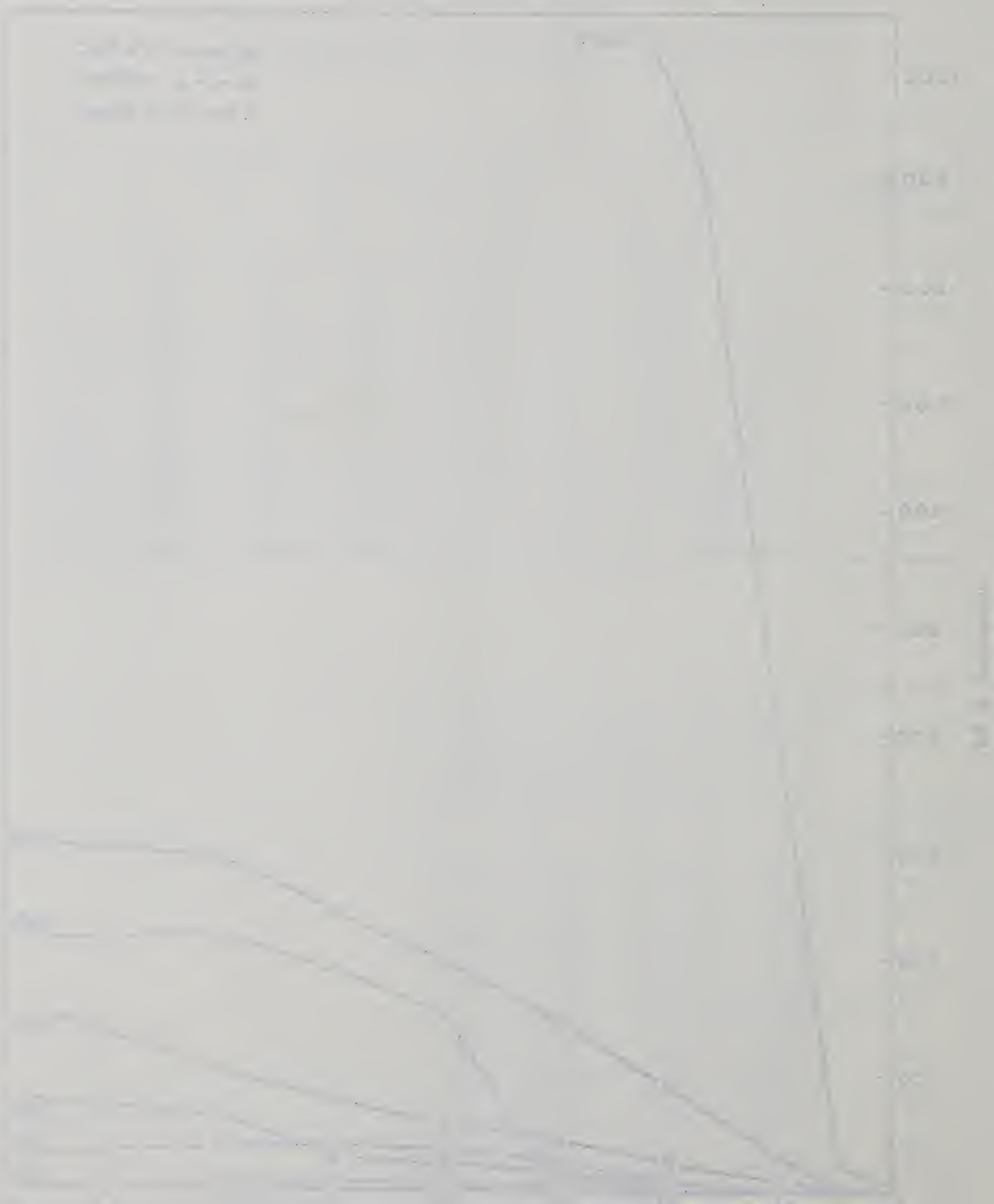
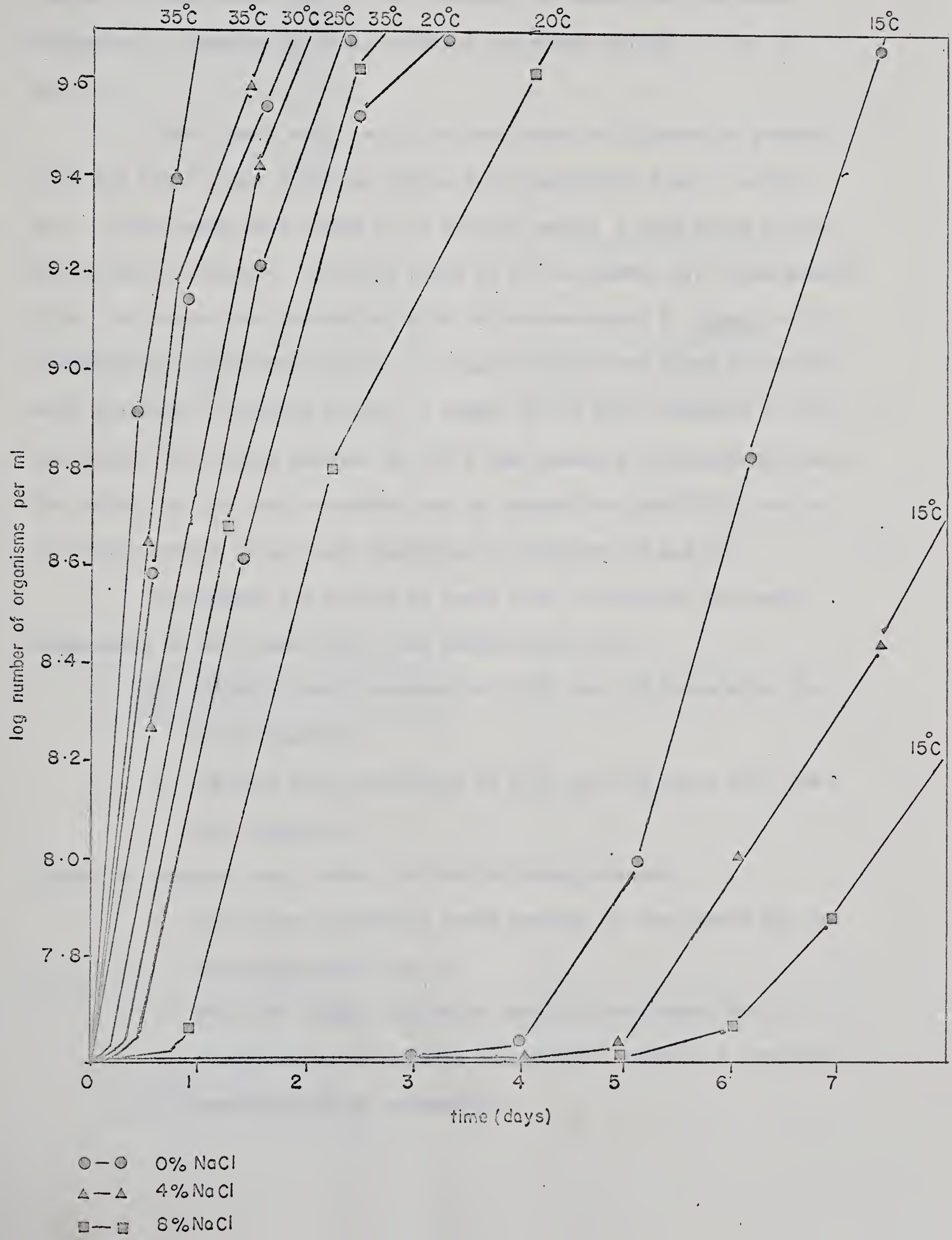


FIG. IX. EFFECT OF CONCENTRATION OF SALT AND TEMPERATURE ON THE MULTIPLICATION OF *S. aureus* Cas 243.



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bands in the acrylamide gel columns. Samples showed variation in the number of bands according to the treatment to which they had been subjected. Examples of this variation are shown in Fig. X - a, b, and c.

Three bands which could be attributed to diffusable products from the brain heart infusion broth, were identified from a control gel. These bands were named 5, 10 and 16, number 1 band being at the anode end of the gel. This was shown to be the marker dye, brom-phenol blue. No bands were present on gels of non-sonicated S. aureus cells, merthiolate or buffered saline. One band at position 8 was observed with dialysed, sonicated cells. A sample of 0% NaCl incubated at 35°C and heated for thirty minutes at 100°C was examined electrophoretically. The effect of the heat treatment was to reduce the density of most of the bands except those bands appearing in position 18 and 19.

Throughout the series of tests used to identify the major components of the crude toxin, two samples were used.

A. 0% NaCl broth incubated at 30°C for 138 hours with the test organism.

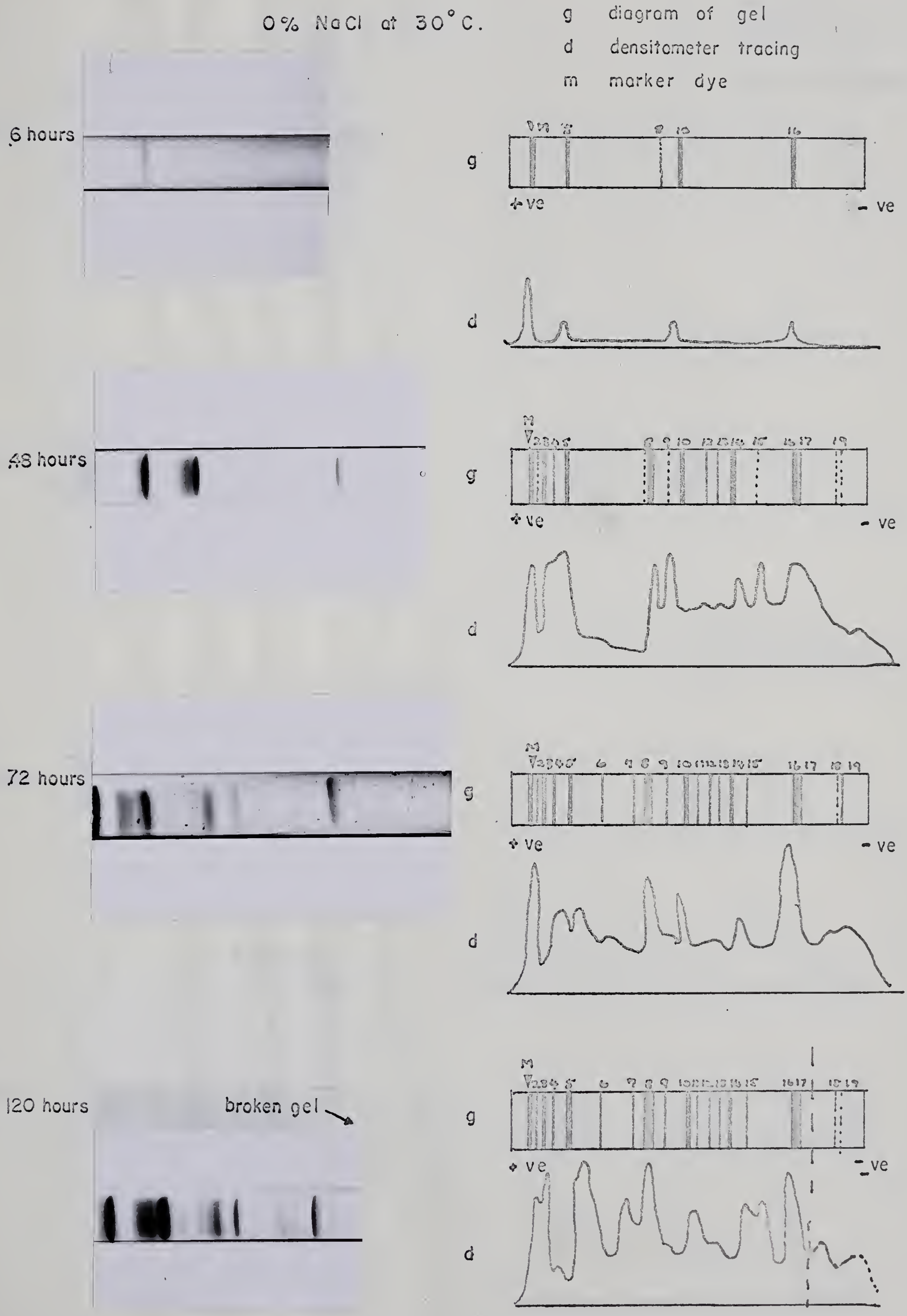
B. 4% NaCl broth incubated at 30°C for 138 hours with the test organism.

These two samples were chosen for the following reasons.

a. Both showed numerous bands present in acrylamide gel on electrophoresis Fig. XI.

b. From the single diffusion tests it was known that B contained no detectable enterotoxin, whereas A contained over 1000 µg of enterotoxin

FIG. X(a). CHANGES IN BAND NUMBER WITH AGE OF CULTURE.



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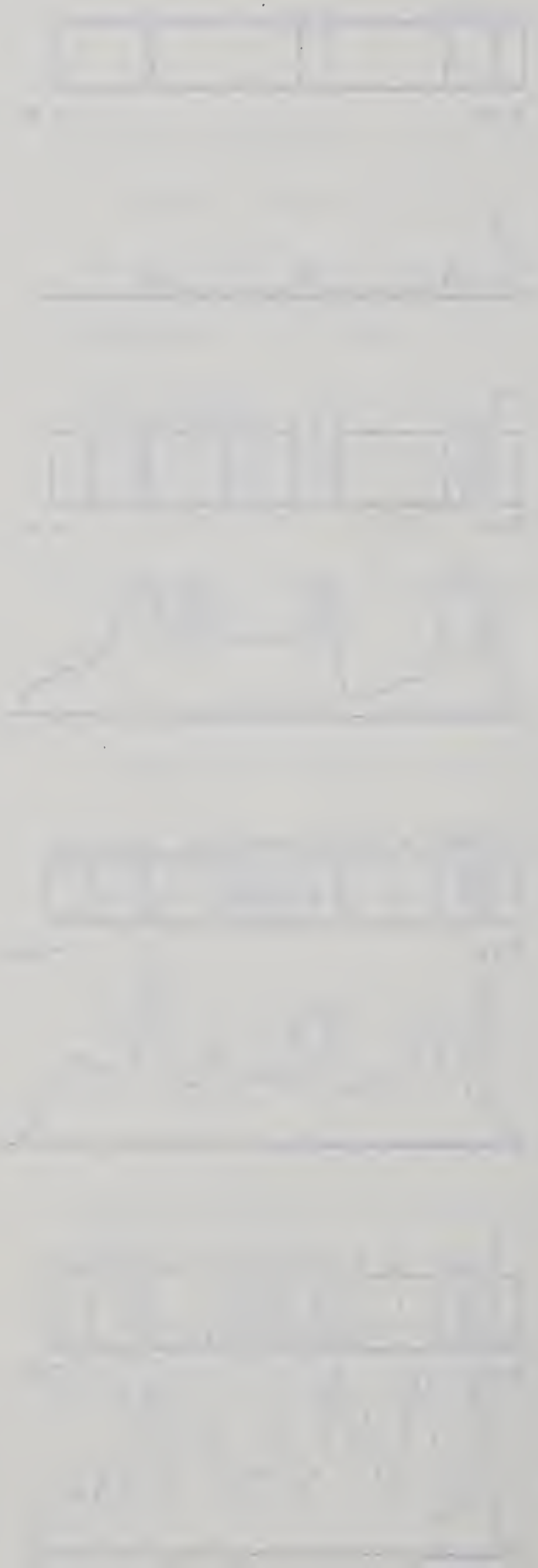
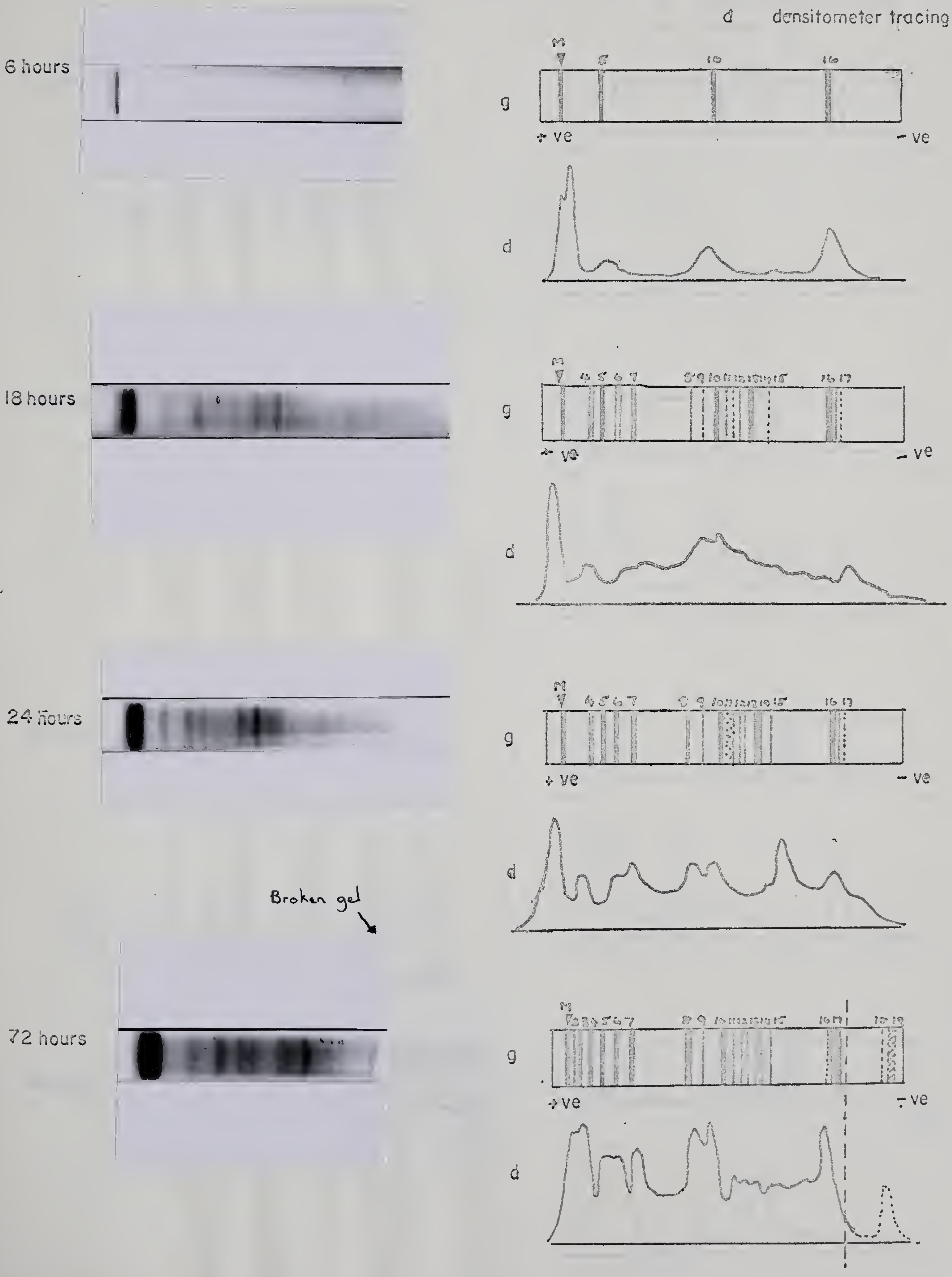


FIG. X(b). CHANGES IN BAND NUMBER WITH AGE OF CULTURE.

4% NaCl at 35°C

m marker dye
g diagram of gel
d densitometer tracing



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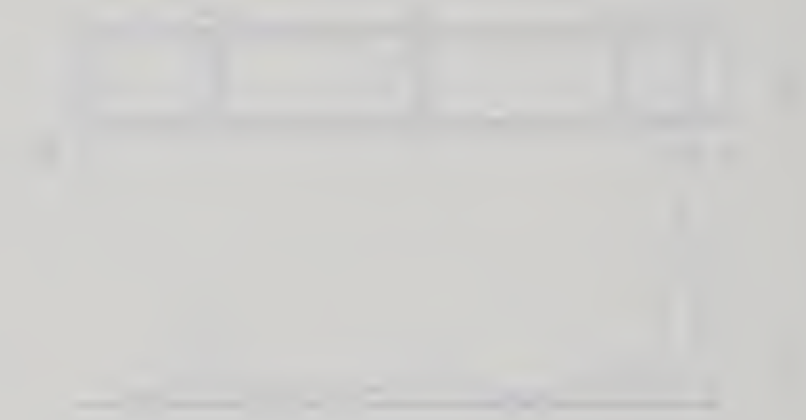

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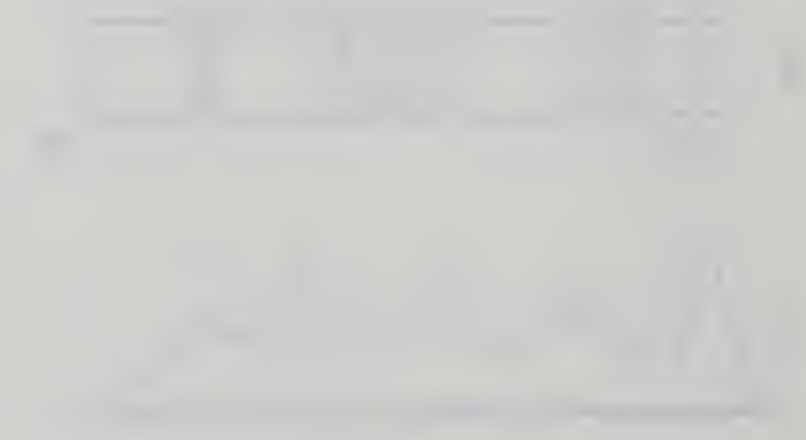

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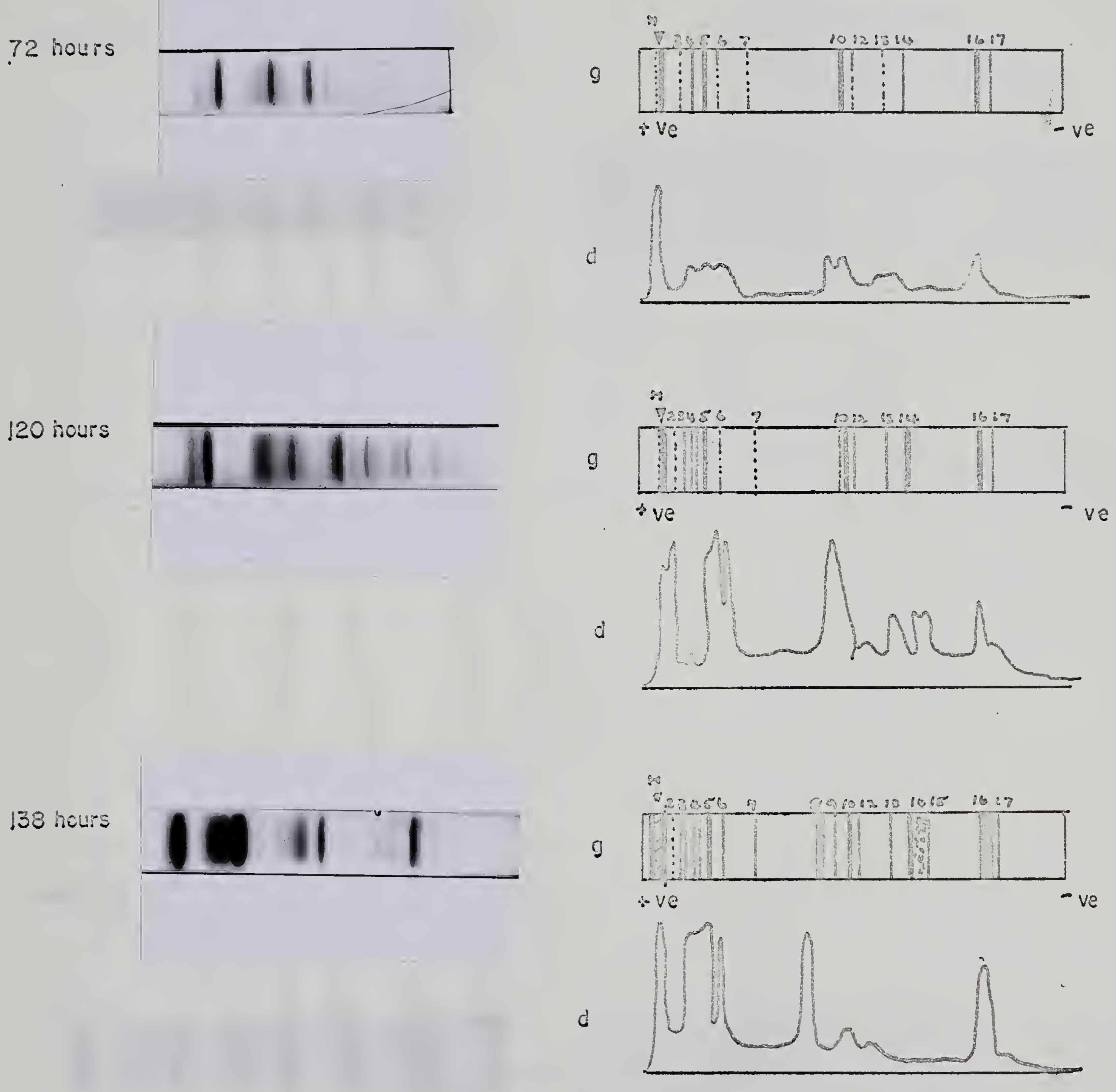
FIG. X (C). CHANGES IN BAND NUMBER WITH AGE OF CULTURE.

4% NaCl at 30°C.

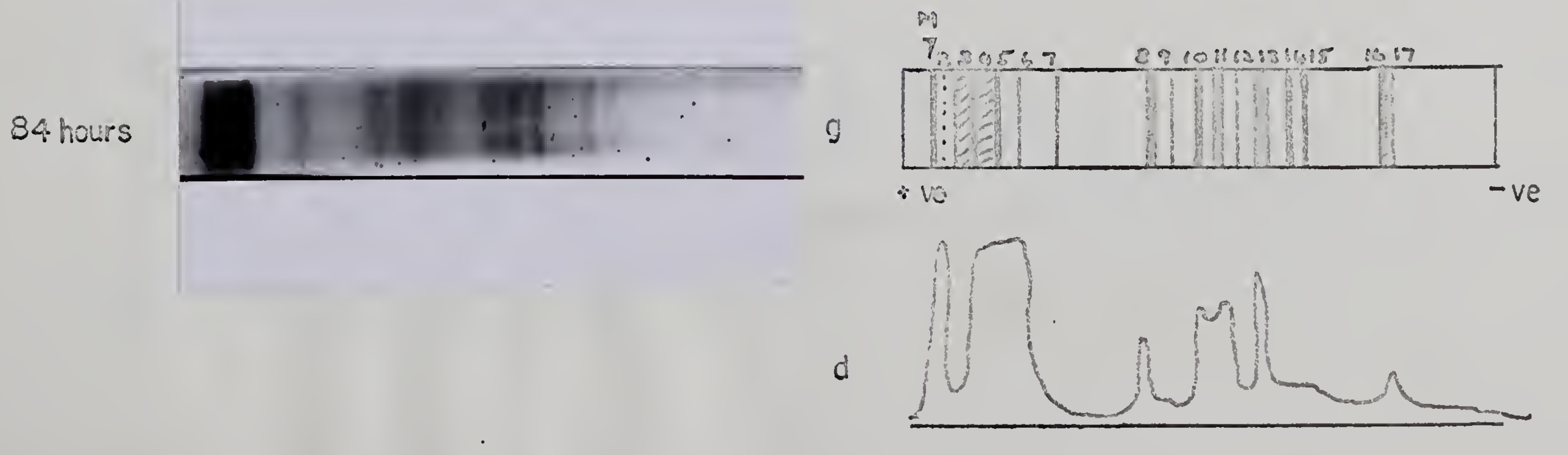
g diagram of gel

d densitometer tracing

m marker dye.



8% NaCl at 35°C



[illegible]

FIG. XI. DISC ELECTROPHORESIS OF SAMPLES

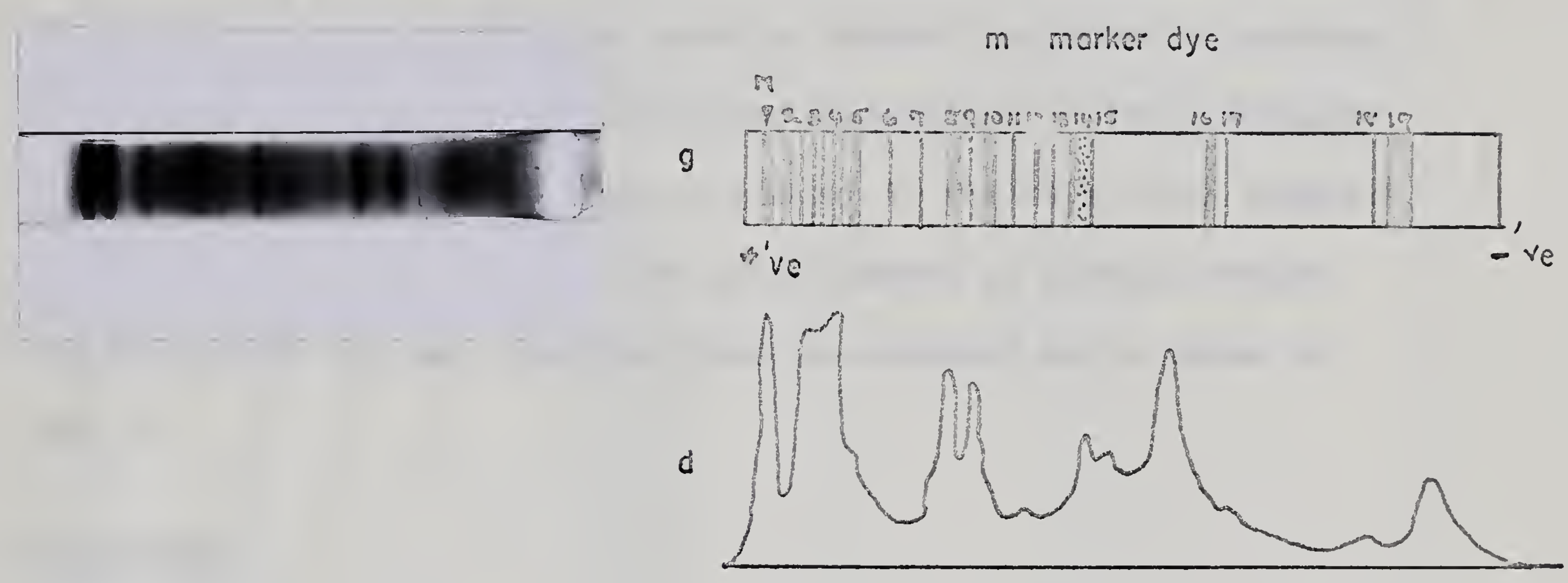
A and B

g diagram of gel

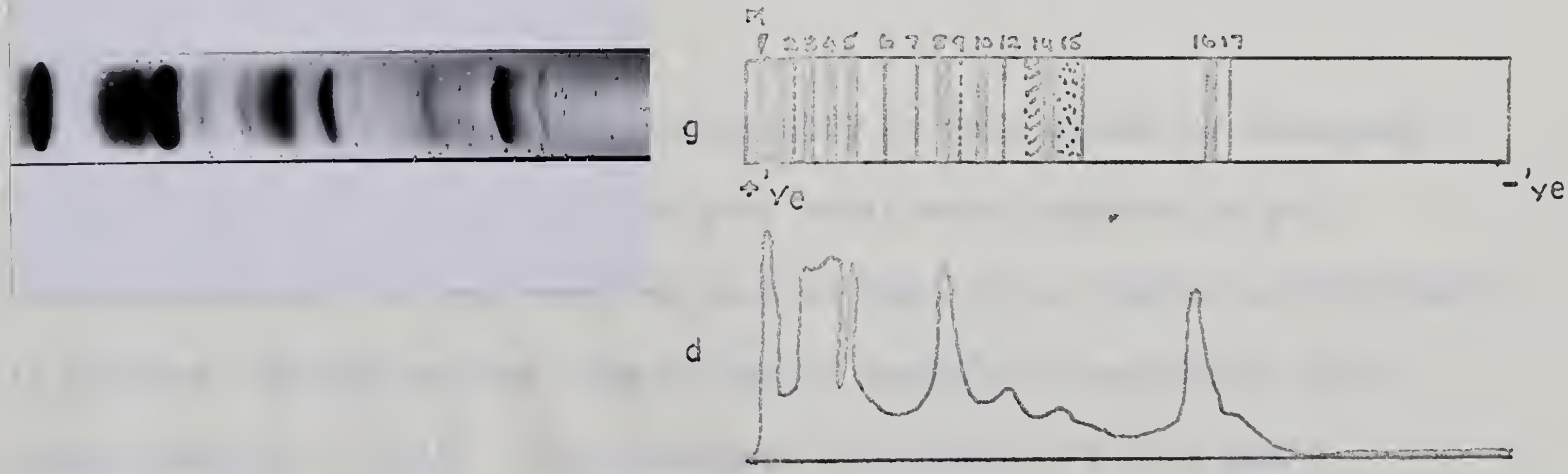
d densitometer tracing

m marker dye

SAMPLE A: 0% NaCl at 30C for 138 hours



SAMPLE B: 4% NaCl at 30 C for 138 hours



Protein

After slicing and eluting the discs from the sample gels the fractions were examined in order to detect the amount of protein per sample. The results obtained from observation of the absorbancy values at 500 mμ for each sample are shown in Tables VII for sample A, and Table VIII for sample B. The actual amount of protein present was calculated for each fraction from the standard curve shown in Fig. VI.

Enterotoxin

The method for identifying the enterotoxin band by a modified slide test has previously been described. Precipitation lines corresponding to those produced by control pure toxin samples were observed in fractions 19, 20, 21 and 22 of sample A. When compared to the stained half of the sliced gel these fractions were equivalent to bands 18 and 19. The observation that these bands were enterotoxin was confirmed when sample B was tested. No precipitation lines were evident in any of the sample fractions, hence again no enterotoxin activity was shown in sample B.

In order to test the sensitivity of this method of detecting enterotoxin, several dilutions of pure toxin were examined by gel electrophoresis. It was observed that no bands were visible or detectable in samples containing less than 133 μg of toxin/ml dissolved in four concentrations of salt. Concentrations of toxin above this limit showed two bands at positions 18 and 19 Fig. XII. This confirmed

Table VII

Absorbancy readings at 500 mμ and equivalent concentrations of protein in fractions of Sample A:- 0% NaCl, 30°C for 138 hours

Fraction No.	Absorbancy reading at 500 mμ.	Protein μg/ml from standard curve
1	0.000	0.0
2	0.002	2.0
3	0.003	2.0
4	0.110	12.0
5	0.138	16.0
6	0.148	17.0
7	0.017	4.0
8	0.006	2.0
9	0.038	6.0
10	0.008	2.0
11	0.018	4.0
12	0.008	2.0
13	0.072	10.0
14	0.008	2.0
15	0.088	11.0
16	0.002	2.0
17	0.000	0.0
18	0.000	0.0
19	0.010	3.0
20	0.002	0.0
21	0.010	3.0
22	0.000	0.0
23	0.000	0.0

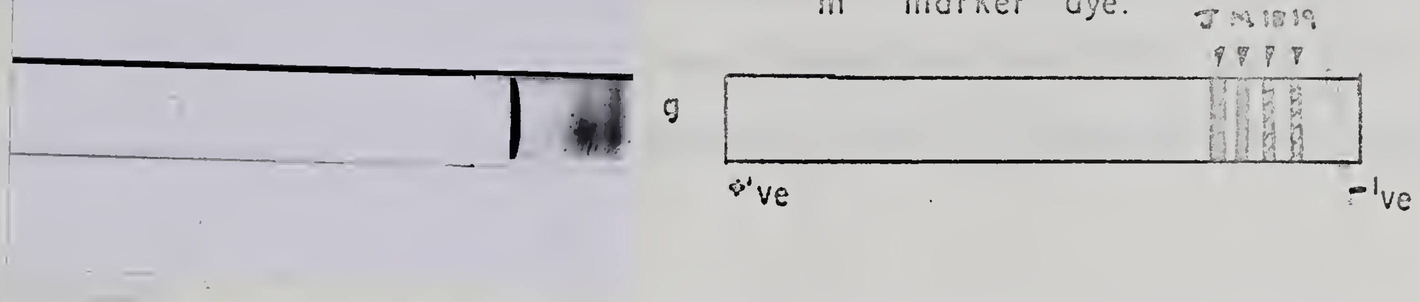
Table VIII

Absorbancy readings and equivalent protein concentration of fractions of Sample B, 4% NaCl, 30°C for 138 hours.

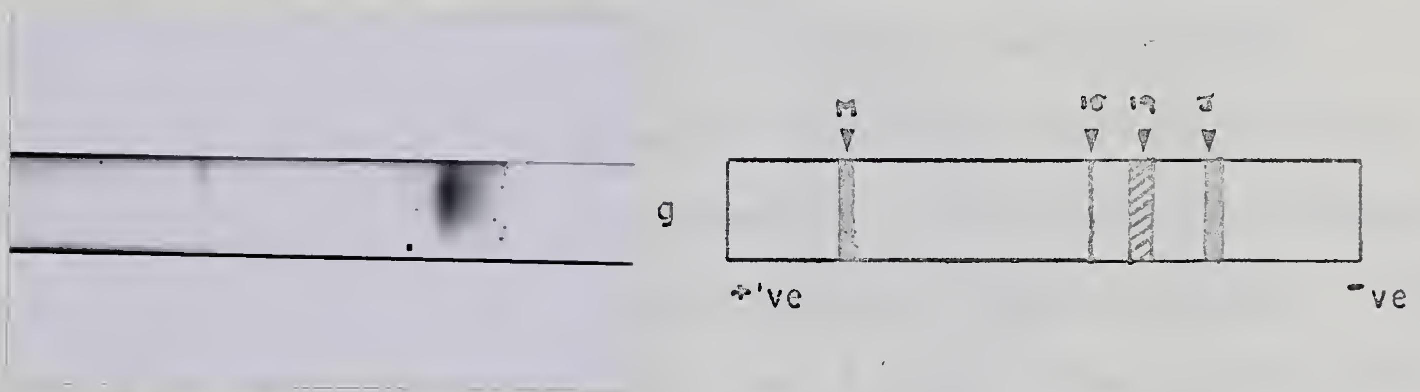
Fraction No.	Absorbancy Reading at 500 mμ	Protein μg/ml from standard curve
1	0.000	0.0
2	0.002	2.0
3	0.045	6.5
4	0.140	12.5
5	0.142	13.5
6	0.020	5.0
7	0.002	2.0
8	0.010	3.0
9	0.010	3.0
10	0.018	4.0
11	0.002	2.0
12	0.035	5.5
13	0.010	3.0
14	0.010	3.0
15	0.072	10.0
16	0.010	3.0
17	0.002	2.0
18	0.000	0.0
20	0.000	0.0
21	0.000	0.0
22	0.000	0.0
23	0.000	0.0

FIG. XII. IDENTIFICATION OF ENTEROTOXIN

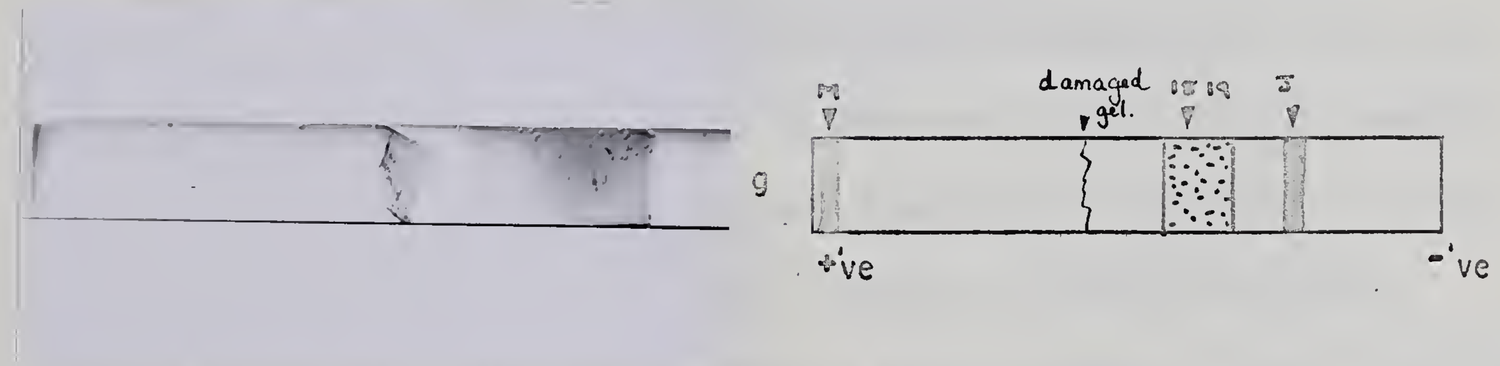
BAND
j junction of large and small pore gels.
g gel diagram
m marker dye.



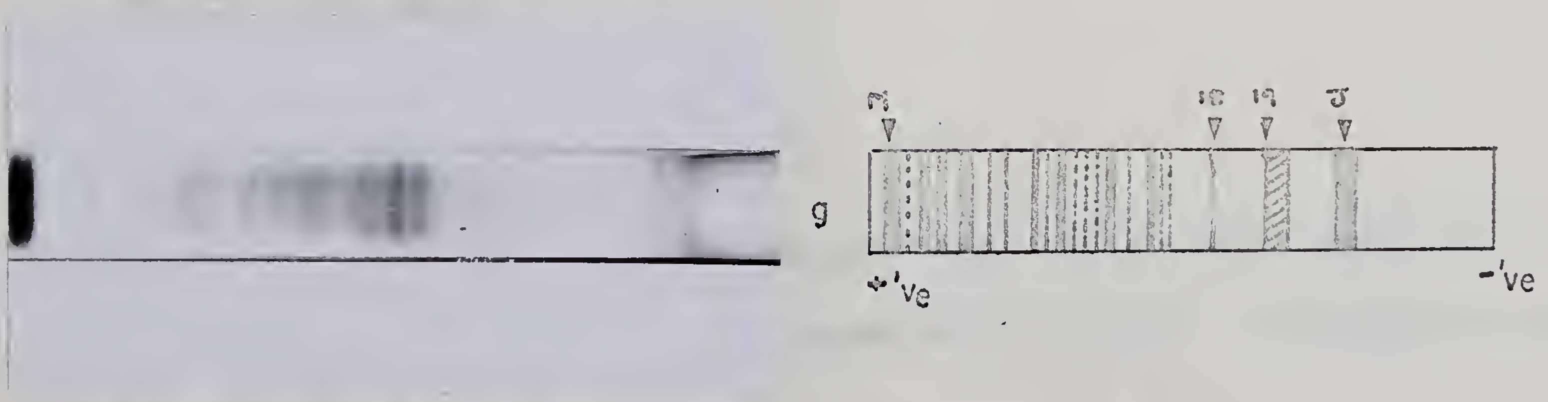
pure toxin in large pore gel.



pure toxin: 400 µg per ml.
in 0% NaCl.



pure toxin: 266 µg per ml.
in 8% NaCl.



0% NaCl at 20 C for 168 hours

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[illegible]

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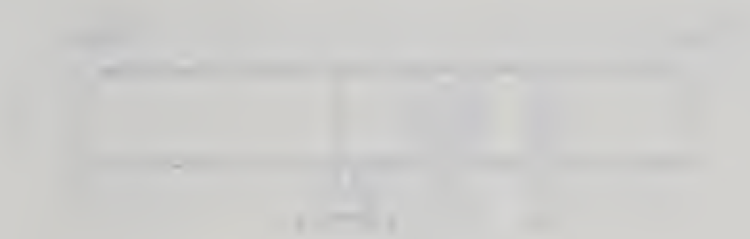

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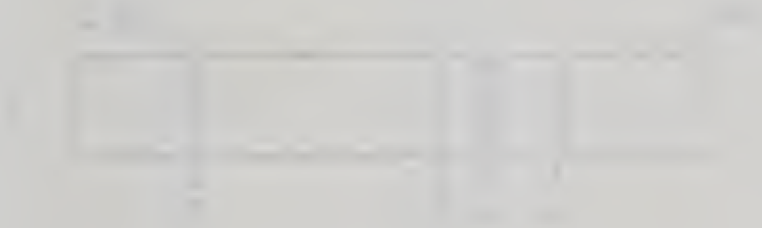

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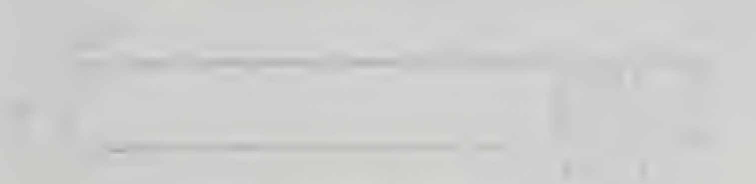

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the observation of other workers studying the electrophoretic mobility of purified toxin on starch, who have found purified toxin splitting into two bands both giving precipitation lines with specific antiserum.

Hemolysins

Fractionated samples of A placed in sterile assay cups on rabbit blood agar and incubated at 37°C for 48 hours showed clear rings of hemolysis around cup numbers 12 and 13 (see Fig. XIII), and also around cup numbers 11 and 14, indicating that bands corresponding to these fractions were α hemolytic. Four different fractions of α hemolysin have previously been observed by density gradient electrophoresis and have been named a, b, c and d (113). These could possibly correspond to bands, 14, 13, 12 and 11 respectively on the gels.

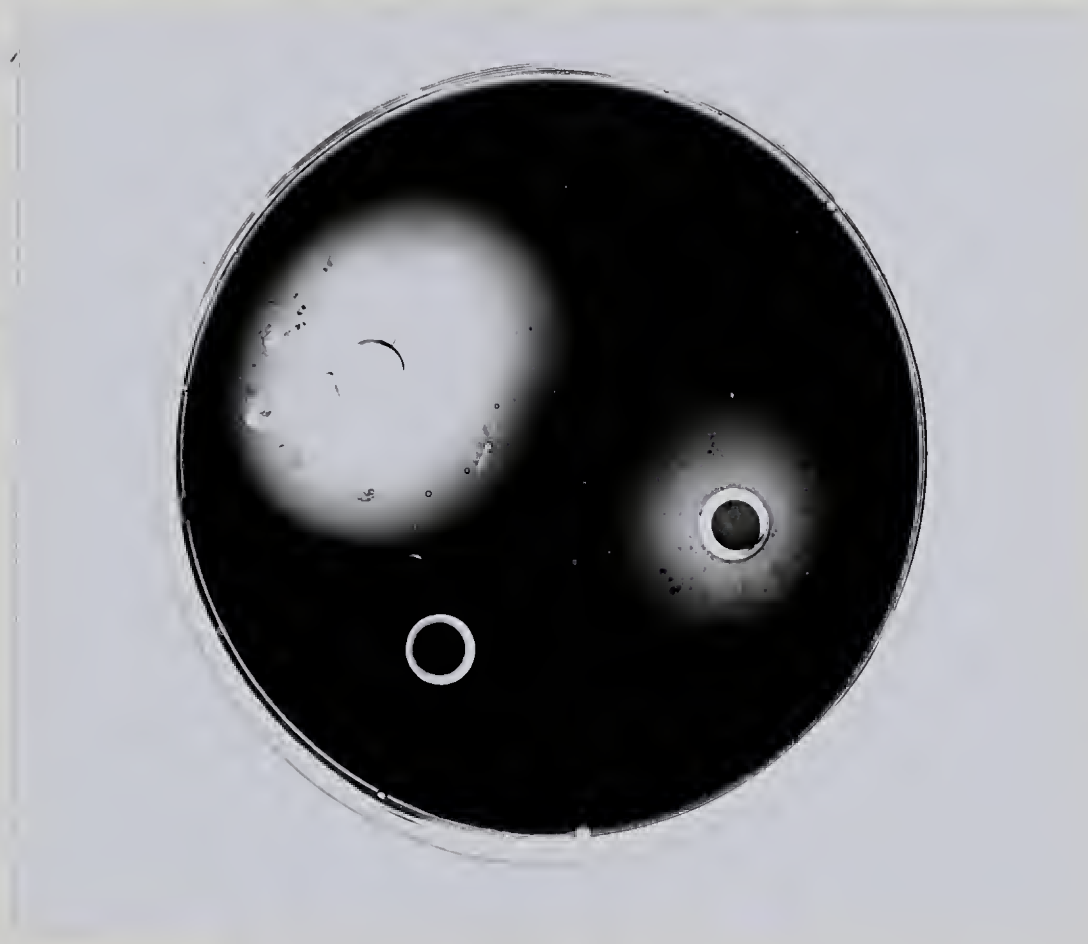
Similar examination of fractions from sample B revealed hemolytic activity in fractions 12 and 13, corresponding to bands 12 and 14 in the gels. Bands 11 and 13 were not visible in this sample.

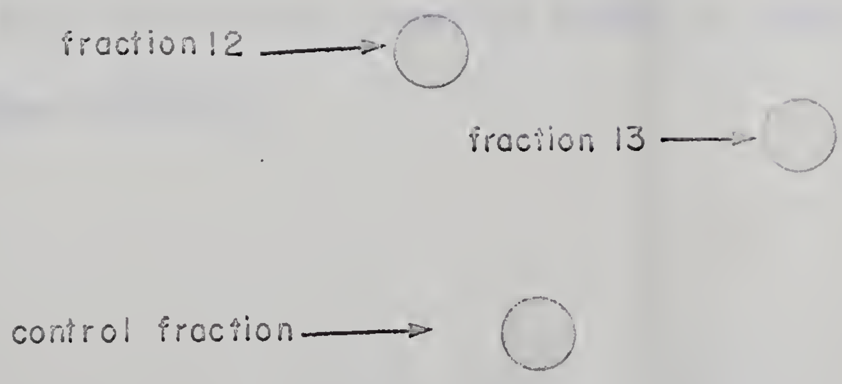
Fractionated samples of A and B were also incubated on sheep blood agar at 37°C and then at 4°C. Zones of clearing were seen around fractions 14 and 15 of sample A and in sample B around fractions 13 and 14. In both instances these fractions correspond to bands 14 and 15 in the gels.

Deoxyribonuclease and Phosphatase

When the deoxyribonucleic acid agar plates were examined for nuclease activity several clear areas were observed. In Sample A, clear

FIG. XIII. ALPHA HEMOLYSIS ON RABBIT BLOOD AGAR PLATE





rings occurred in the agar around fractions 7, 8, and 9 Fig. XIV. In sample B faint rings could be detected around fractions 8 and 9. It is known that lysing of the cells of staphylococci occurs during rapid growth. The increase in nuclease activity with time is evident from these two samples and the observation of a band present in sonicated cell samples at position 8 would seem to indicate that this band represents deoxyribonuclease.

Phosphatase activity, indicated by a blueing of the clear rings was not evident. However a marked blueing occurred in the agar around fractions 4 and 5 in both samples A and B, indicating possible phosphatase activity in bands 2 or 3 of the gels.

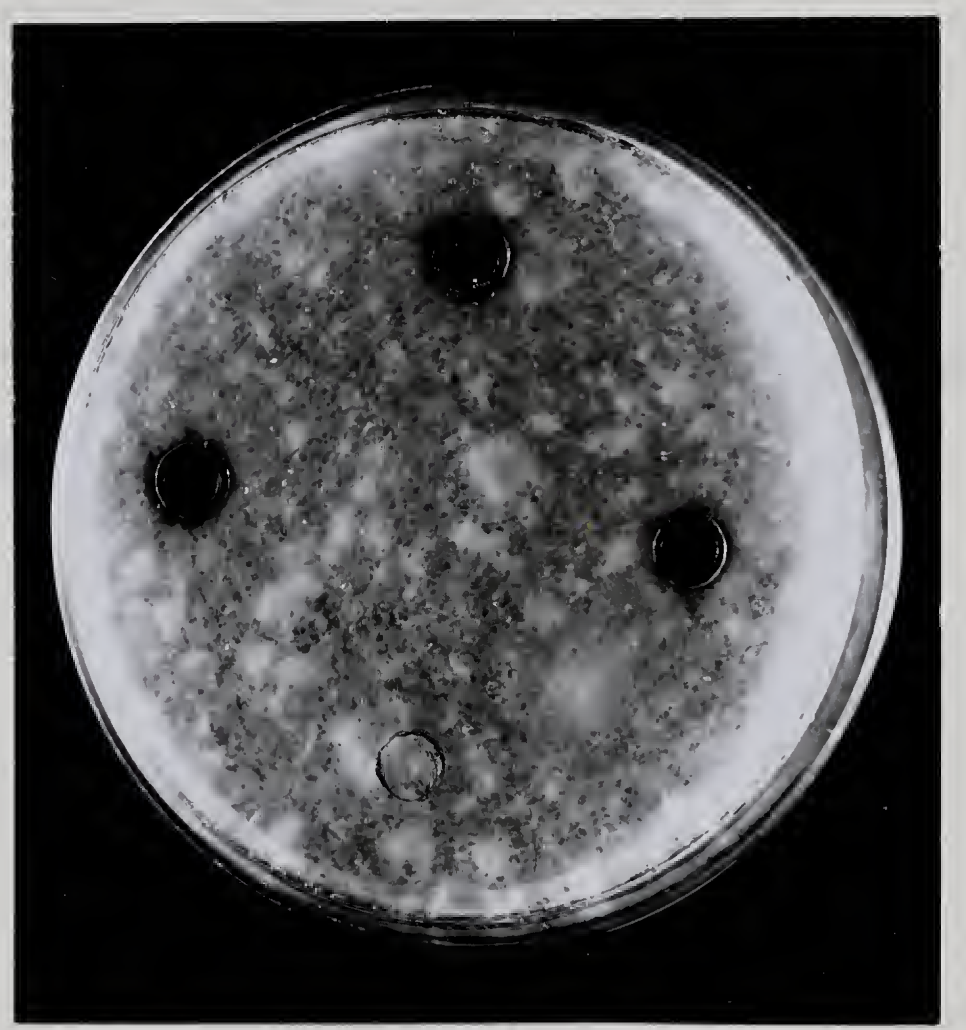
Lipase

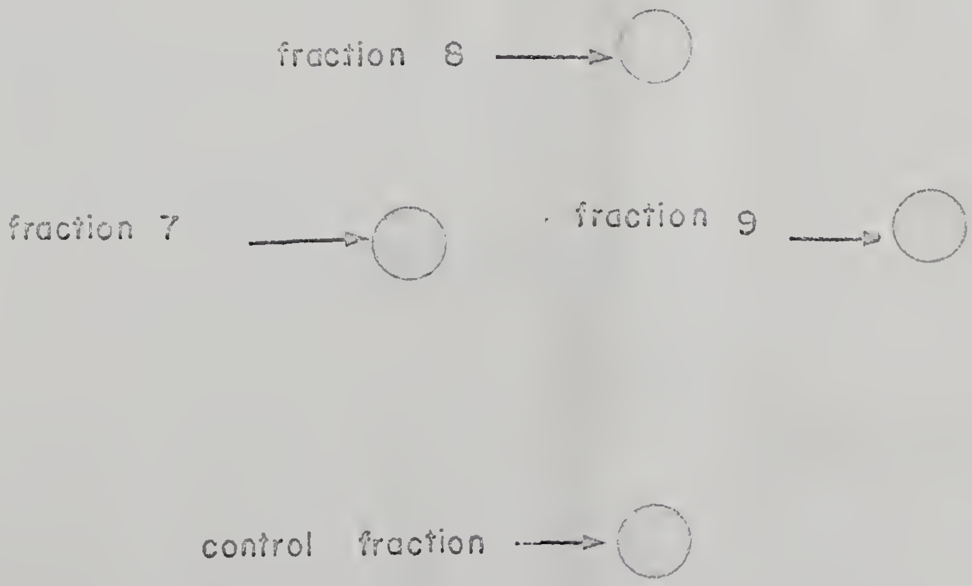
Clear zones surrounding fractions 6 and 7 in both samples A and B when samples were incubated on tributyrin agar indicated lipase activity. Therefore band number 4 is most probably the position for lipase.

Hyaluronidase

A positive clot formation was observed in both samples A and B, in all fractions but numbers 8, 9, 10 and 11. This indicated the presence of hyaluronidase in these fractions. A band corresponding to this activity is possibly number 7, this being the region of maximum enzyme activity.

FIG. XIV. DEOXYRIBONUCLEASE ACTIVITY ON AGAR ASSAY PLATE



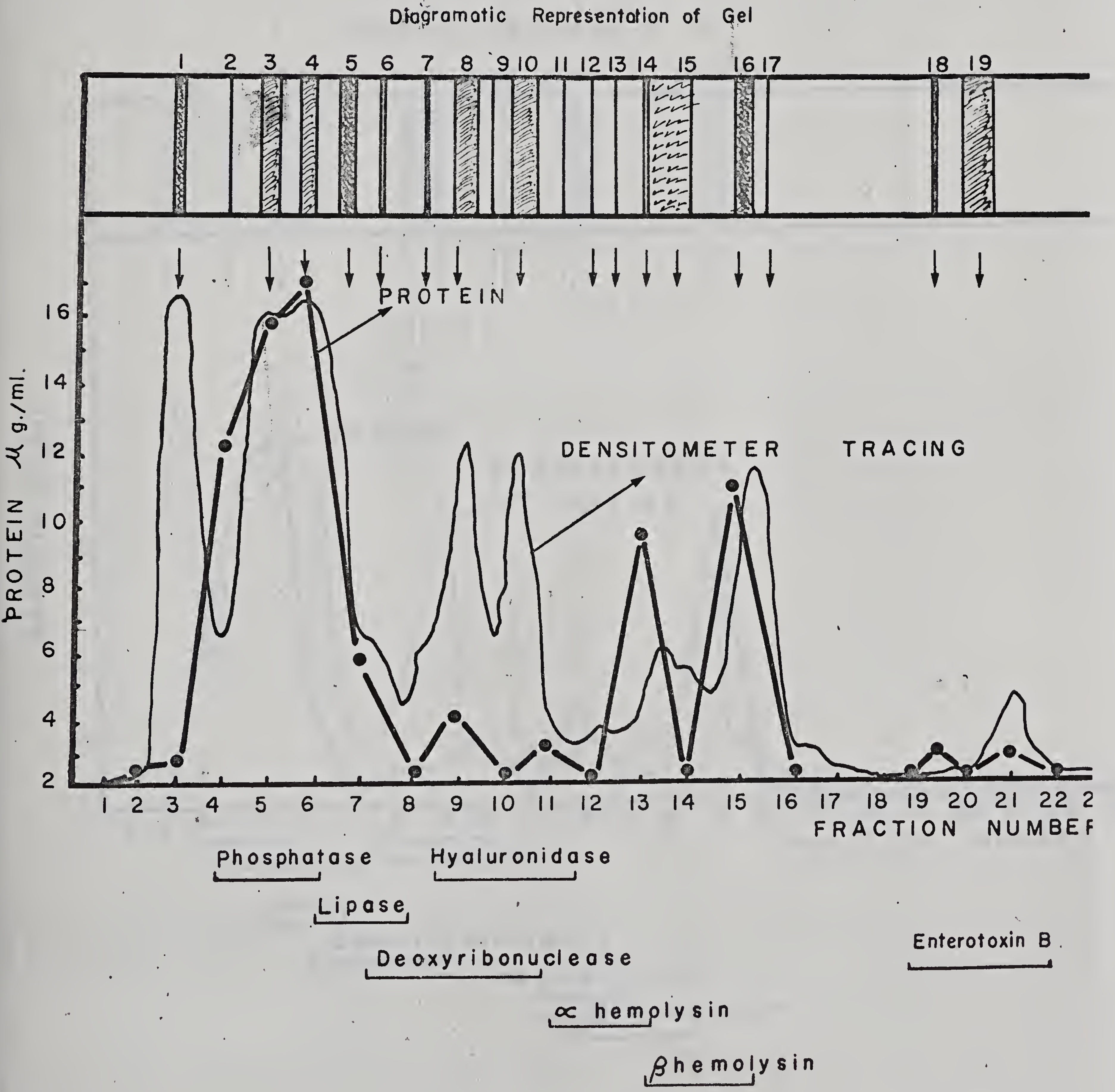


Coagulase

In agreement with other workers (114) no coagulase activity could be detected in any of the fractionated samples. Only whole staphylococcal cells produced activity, indicating that the ability to coagulate plasma is a characteristic of whole cells.

Composite diagrams Figs. XV and XVI illustrating the information obtained from this series of tests were constructed. These show the electrophoretic mobility of the various extracellular products of S. aureus var. Cas. 243.

FIG. XV THE ELECTROPHORETIC MOBILITY AND PROTEIN CONTENT OF CRUDE EXOTOXIN PRODUCED BY *S. aureus* Cas 243 INCUBATED IN 0% SODIUM CHLORIDE BROTH FOR 138 hours at 30°C



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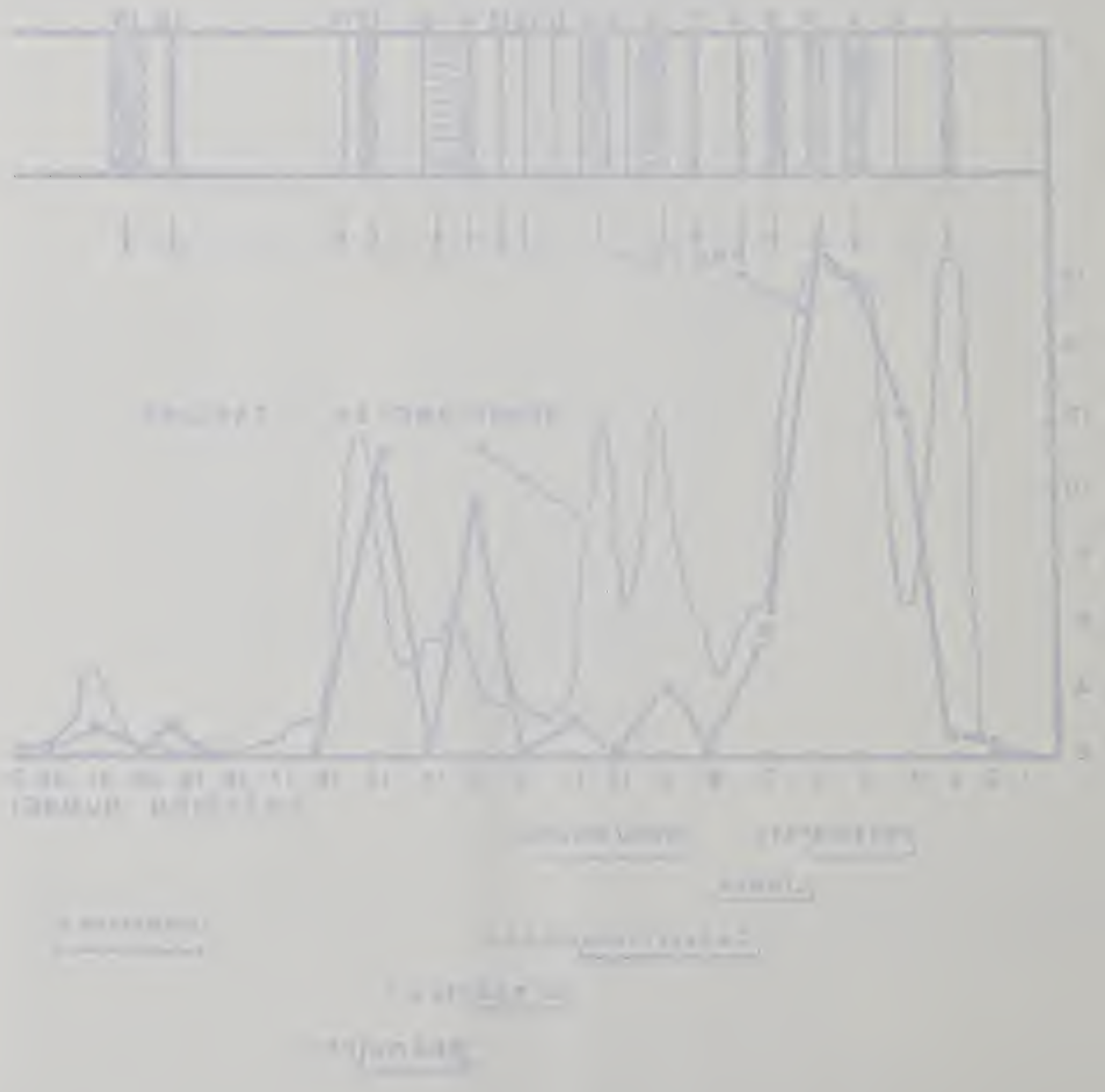
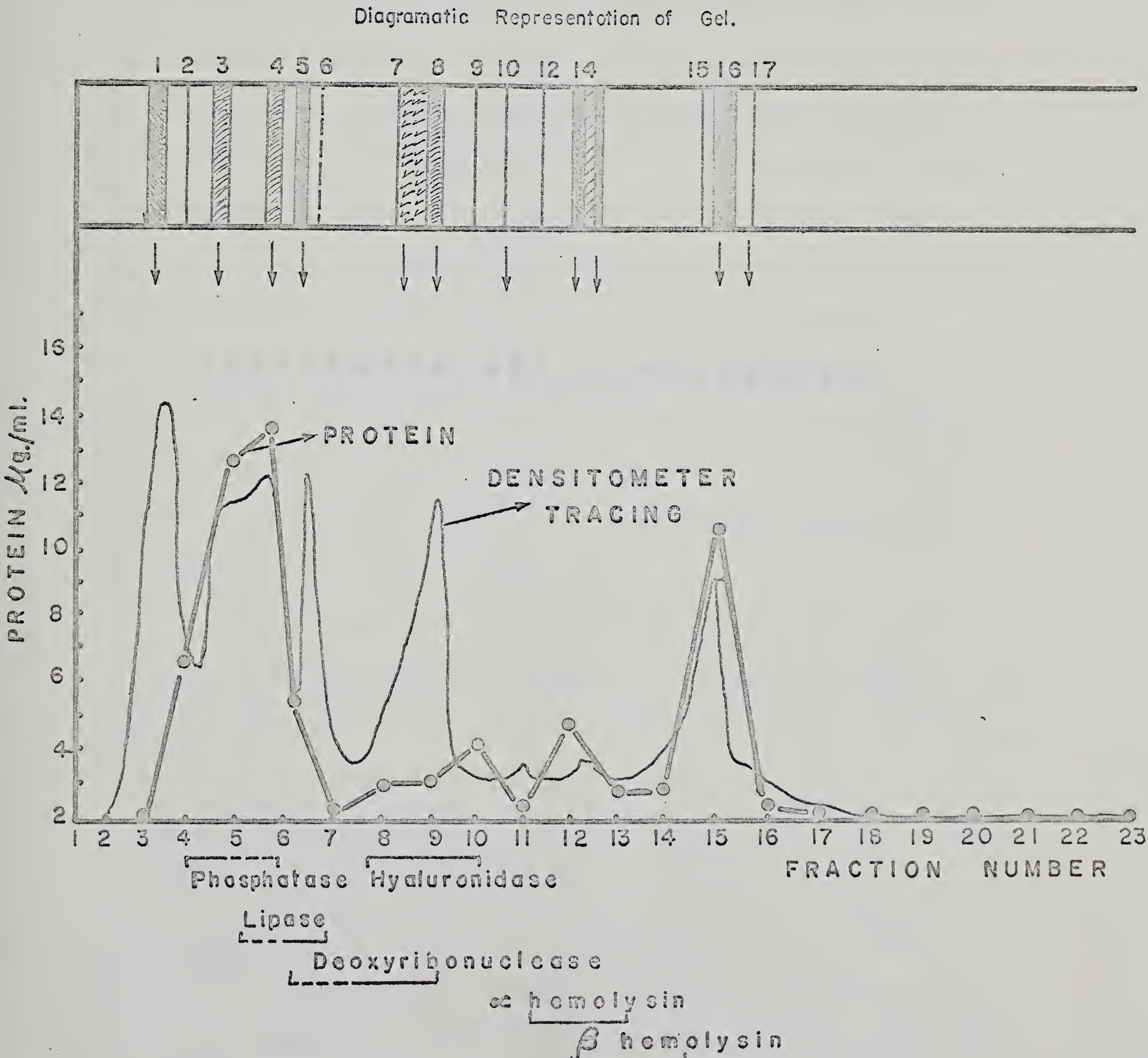


FIG. XVI. THE ELECTROPHORETIC MOBILITY AND PROTEIN CONTENT OF CRUDE EXOTOXIN PRODUCED BY S. aureus Cas 243 INCUBATED IN 4% SODIUM CHLORIDE BROTH FOR 138 hours at 30°C



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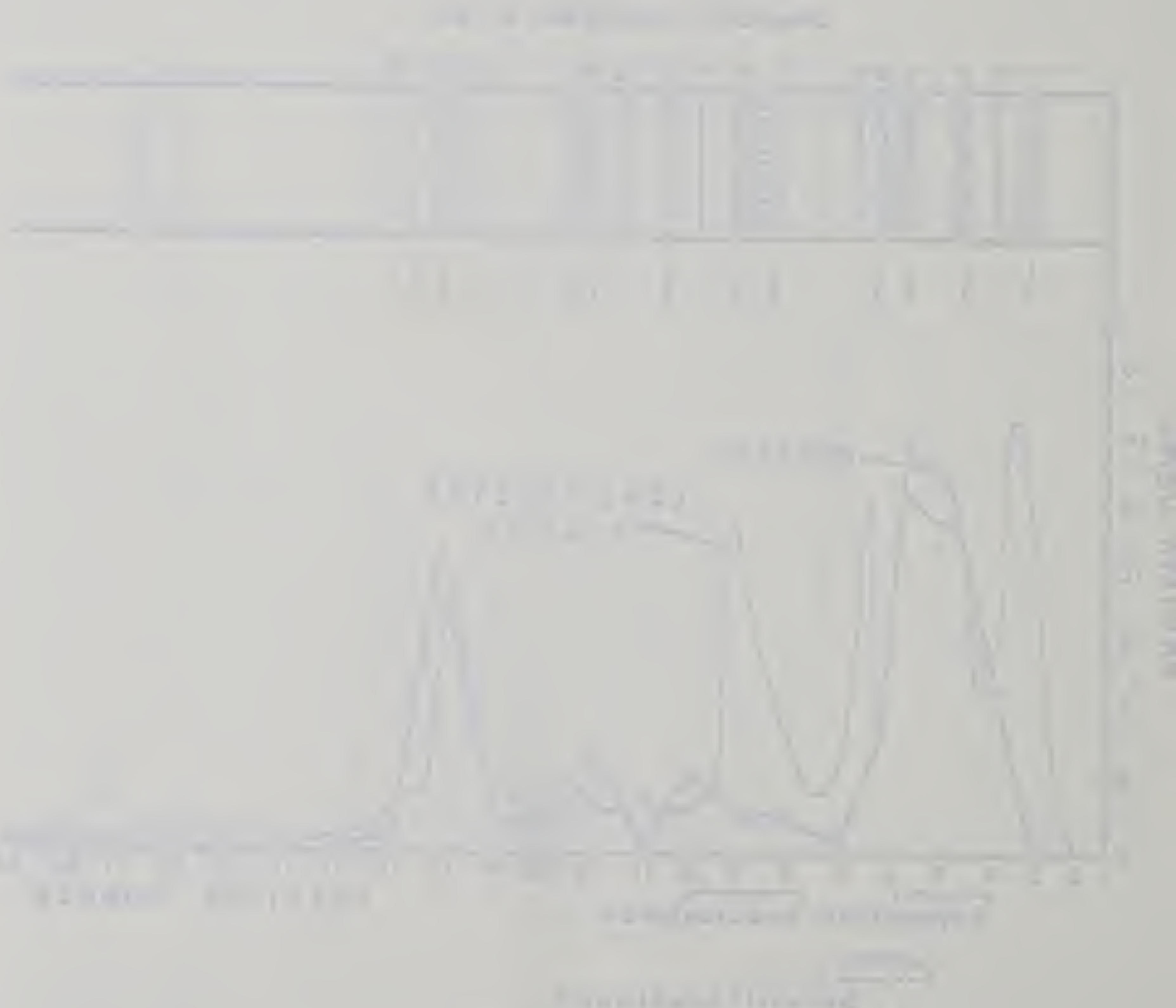


DISCUSSION AND CONCLUSIONS

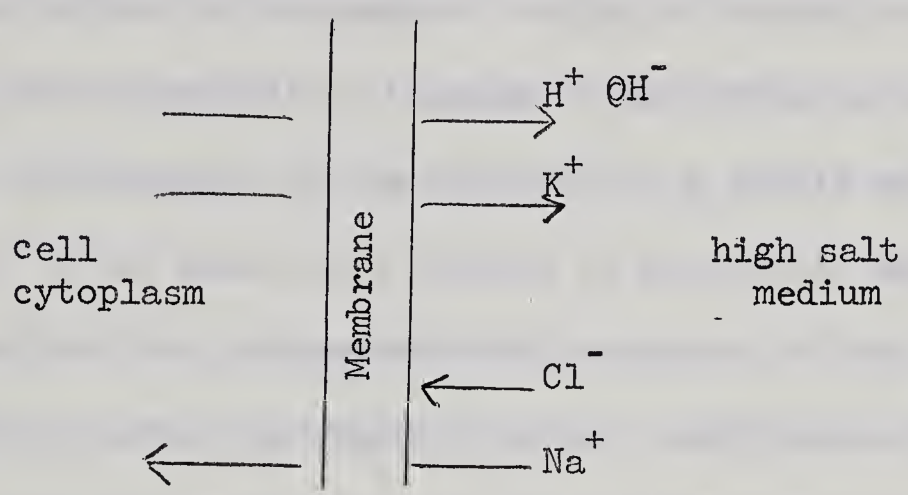
DISCUSSION AND CONCLUSIONS

Elek (115), has stated that 'the medium, conditions of incubation, and times of harvesting, are known to affect the nature and ratio of the various extracellular products of staphylococci.' The literature pertaining to the effect of environmental conditions on the growth, and exotoxin production of staphylococci associated with food poisoning, is relatively scant. It was the object of this investigation to provide more information on this subject. Sodium chloride, time and temperature of incubation were selected as suitable variables because of their significance in the food industry.

The results obtained for growth of S. aureus Cas. 243 in broth cultures, show that the organism is able to multiply in 12% sodium chloride between the temperatures 20°C and 35°C. Numbers of organisms formed in these growth experiments were high enough, theoretically, to produce an emetic dose of enterotoxin.

When the amount of enterotoxin produced from cultures used in these growth experiments was examined, it was found that enterotoxin production did not, in most cases, run parallel to growth. The exception to this was in 0% sodium chloride broth at 35°C. Toxin was produced in the stage of rapid growth, the logarithmic phase. Production of toxin was inhibited in broths containing 4% and 8% sodium chloride at all temperatures below 35°C and at all temperatures in the 12% broth. From these results it can be concluded that growth and exotoxin production are depressed by increasing concentrations of sodium chloride. This effect is greater on toxin production than growth.

To explain this phenomenon more detailed investigations would be necessary. However we can examine a few possibilities. Diagrammatically the situation can be represented in terms of passage of ions through the semipermeable membrane in cells growing at high salt concentrations. Water will pass under osmotic pressure from the cell to the medium, whereas sodium ions will pass easily through the cell membrane into the cell. Chloride ions have difficulty in passing through the membrane and remain at the cell surface (116). The increase in the sodium ion content in the cell will cause an increase in the flow of potassium ions out of the cell. The effect of these changes in ion concentrations within the cell, obviously have some effect on the amount of enterotoxin present outside the cell as an extracellular product.



Three possible explanations could account for this lack of detectable toxin in the cultures growing in high salt media.

a. The toxin was prevented from being formed within the cell.

b. The toxin was formed but in some way altered, before being passed out of the cell with a loss in biological properties.

c. The toxin was formed, but could not pass out of the cell.

We may consider each of these possibilities in connection with the change in ion concentrations inside and outside of the microbial cell.

A loss of water from the cell could prevent toxin formation by an inhibitory effect on the mode of action of specific enzymes concerned with toxin synthesis. Change~~s~~ in the structure of the protein itself is unlikely, as the molecule is a simple and fairly stable protein. If by chance the protein is altered in any way, such changes could affect the antigen-antibody reaction so that toxin would be undetectable by normal serological tests. Dehydration could also affect the permeability mechanisms of the cell membrane, by some change of the transport systems. Action on a specific enzyme could prevent toxin transport to the outside medium.

It is known that an increase in the sodium ion concentration within a cell can affect the growth of the cell by increasing the length of the lag period. This is due to the effect of sodium ions on the enzymes associated with respiration in the cell (117). It is possible that such an effect could apply also to those enzymes concerned with toxin production. It is unlikely that sodium ions would have an effect on actual protein structure as sodium ions are known to act on proteins in a stabilising, rather than a disrupting, manner. The effect of sodium ions upon the permeability mechanisms seems the most feasible explanation for the lack of detectable enterotoxin at high concentrations of sodium chloride. It has been established

that sodium ions have an inhibiting effect on one of the enzymes involved in transport within the cellular membrane (118). It is possible that this form of inhibition is not specific to this one enzyme, adenosine triphosphatase, but applies also to a specific enzyme or enzymes involved in toxin transport. A general blocking of the whole permeability mechanism in this way could prevent toxin, after formation, from being transported to the outside medium. Further experiments investigating the enterotoxin content of cell free extracts would be needed to confirm this theory.

The presence of an accumulation of chloride ions at the cell surface could possibly upset the metabolic balance involved in toxin production.

Experiments using Escherichia coli to test the effects of osmotic up-shock, have shown that protein and nucleic acid synthesis are temporarily stopped at high salt concentrations although amino acid pool material continued to be incorporated (119). The explanation for these observations was that slow movement of sodium ions into the ribosomes of the cell caused dehydration of these structures to take place and therefore protein synthesis was inhibited. Again, however, applying this to staphylococcal toxin production, growth would also be inhibited under these conditions. An interesting feature of osmotically shocked cells is that although loss of amino acid pools from the damaged cells may occur, no effect on growth is apparent (120).

The effect of temperature on growth and exotoxin production is quite noticeable from the results. Maximum toxin production occurs

at a temperature of 35°C. (It may be interesting to investigate the toxin production at 40°C in further experiments.) Below 35°C a marked decrease in production occurs at all concentrations of sodium chloride.

The optimum temperature for some enzyme reactions is known to increase with increasing salt concentration (121). If this were the case with enzymes involved in toxin synthesis, the physiological temperature of the system itself may be lower than the actual temperature of incubation. Although the mechanisms of toxin production are unknown, the results obtained in this study may be of practical importance to the food industry. Indications are that storage of foods containing enterotoxigenic staphylococci at temperatures from 20° - 35°C could result in the formation of large amounts of enterotoxin. The addition of sodium chloride (4% or greater) would inhibit toxin formation at temperatures up to 30°C, however, growth of the organism would not be inhibited. As a result of these findings, although coagulase positive staphylococci may be abundant in salted foods, this is not an indication of the presence of toxin, unless the food has been kept at 35°C for up to 24 hours. It is appreciated that other factors associated with the internal environment of specific foods could also have marked effects on growth and toxin production.

The use of the technique of disc electrophoresis was investigated as a possible alternative to serological and animal tests for enterotoxin detection. Under the conditions of pH and gel pore size used in the experiment the sensitivity of the test was lower than

serological tests. However, it is possible that further experimentation with different pH and pore sizes could increase the sensitivity and provide a relatively simple test for detection of enterotoxin.

Electrophoresis of the crude exotoxin obtained from growth of staphylococci allowed an investigation of the electrophoretic mobility of the various components of the exotoxin. The effect of salt concentration, time and temperature of incubation on the production of these components, was possible after identification of several of the bands.

The maximum number of bands obtained in the gels was greater than has previously been observed by starch and paper electrophoretic methods, emphasising the sensitivity of the disc-gel technique (13). In general, the positions of the various components obtained from electrophoresis of the crude toxin agreed with results obtained by these methods and data from column chromatography of the exotoxin (114).

On examination of the number of bands present in the gel it was seen that during the first few hours of growth few, if any, extracellular products were detectable. With increasing multiplication of the cocci during the log phase, up to nineteen bands appeared. Investigating the presence of some of the known bands more thoroughly the approximate time of production and the length of time of production could be estimated. The effect of the three variables on enterotoxin production has already been discussed. Among the first products to be detected at higher temperatures and low salt concentration were the hemolysins, alpha-hemolysin usually before beta-hemolysin. The number of bands associated

with α hemolytic activity tended to vary according to the age of the sample, the numbers increasing up to four. The width of the bands formed did not change with age. This would indicate that hemolysin is produced only during the early stages of multiplication.

Few bands were detectable in gels of exotoxin produced in 12% salt at any temperature. Only the region corresponding to deoxyribonuclease activity was prominent, probably due to increased lysis of cells growing at high salt concentrations. The density of this region also increased with age in all other cultures. It is known that an increased concentration of sodium ions in the cell causes the nuclease to detach from its locus on the ribosomes and autolytic reactions may commence, leading to lysis (123).

Hyaluronidase appeared to be formed after about three days incubation at the higher temperatures and the lower salt concentrations. The band increased in density for up to one week of growth.

Summarising, the major effect of sodium chloride at the lower concentrations was to inhibit production of enterotoxin, increase the amount of deoxyribonuclease in the exotoxin, and delay the production of the hemolysins. The effect of lower temperatures appeared to be a reduction in growth, and a decrease in the amount of extracellular products produced by the cell.

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